

Molecular Characterisation of GnRH Regulated Factors Isolated From Gonadotroph Cells

Lynda Chang
BSc (Hons) Dundee University

Medical Research Council
Human Reproductive Sciences Unit
Centre For Reproductive Biology
37 Chalmers Street
Edinburgh
EH3 9ET
UK

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In loving memory
of Dad.

Declaration

I declare that all the experiments detailed in this thesis were the unaided work of the author except where acknowledgement is made by reference. No part of this work has previously been accepted for any other degree, nor is any part of it being concurrently submitted in candidature for another degree.

Lynda Chang

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Abstract

The pituitary gonadotrophins luteinising hormone (LH) and follicle stimulating hormone (FSH) are differentially secreted from the gonadotroph cells of the anterior pituitary in response to gonadotrophin releasing hormone (GnRH). Both LH and FSH are members of the glycoprotein hormone family and are comprised of a common alpha glycoprotein subunit (α GSU) and a function specific beta subunit (LH β or FSH β). GnRH also differentially regulates all three genes; however, the factors that mediate this have yet to be fully elucidated. The L β T2 cell line recapitulates a differentiated gonadotroph cell and was used as a model system for differential display RT-PCR analysis (DD-RT-PCR) of GnRH regulated transcripts. These transcripts were characterised and are described in this thesis.

DD-RT-PCR showed that different pulse regimes of GnRH differentially regulated gene expression and identified rapid changes in mRNA transcript levels in response to GnRH. Both myosin light chain and a putative tyrosine phosphatase were upregulated by GnRH. Other transcripts included two expressed sequence tags (ESTs), one expressed in the testes, the other in the mammary glands of pregnant or lactating mice. In addition GnRH also down-regulated Fanconi's anaemia complementation group A (FAA) mRNA expression levels.

Fanconi's anaemia (FA) is an autosomal recessive human disease, characterised by aplastic anaemia, short stature, developmental abnormalities, microcephaly, and infertility. The majority of FA genetic abnormalities map to the FAA gene,

which is thought to have a role in DNA repair, and cell cycle checkpoint control. The role of FAA in controlling gonadotrophin gene expression was investigated by transient transfection assay in L β T2 and α T3-1 gonadotroph cells. α T3-1 cells represent gonadotrophs from an earlier developmental stage compared with L β T2 cells and only synthesise α GSU. The results indicated that FAA specifically repressed the GnRH response of the α GSU promoter in L β T2 cells. The regulatory region of the FAA mRNA was mapped to between 1bp and 965bp, which encodes the region on the FAA protein between amino acid (aa) residues 1aa and 322aa. The results also suggest that FAA may mediate its effects through a paired homeodomain binding site on the α GSU promoter. Therefore DD-RT-PCR has identified a number of mRNA transcripts that are regulated by GnRH. These may have roles in secretion or second messenger signalling pathways. Furthermore, the discovery that GnRH regulated FAA mRNA levels, has identified a novel role for FAA in regulating α GSU transcription.

Abbreviations

A	adenine
aa	amino acid
ACTH	adrenocorticotrophin
AcCoA	acetyl coenzyme A
α GSU	alpha gonadotrophin subunit
ANOVA	analysis of variance
AP-1	activator protein 1
AS	antisense
ATP	adenosine triphosphate
BMP	bone morphogenic protein
bp	base pairs
BSA	bovin serum albumin
C	cytosine
$^{\circ}\text{C}$	degrees Celsius
C-terminus	carboxy terminus
Ca^{2+}	calcium
CAT	chloramphenicol acetyltransferase
cAMP	cyclic 3',5' adenosine monophosphate
cDNA	copy DNA
cfu	colony forming units
CG	chorionic gonadotrophin
CgA	chromogranin A
CgB	chromogranin B
Ci	Curie
CMV	cytomegalovirus
CNS	central nervous system
cpm	counts per minute
CPHD	combined pituitary hormone deficiency
CRE	cAMP response element
DAG	diacylglycerol
DD-RT-PCR	differential display reverse transcriptase polymerase chain reaction
DDT	dithiothreitol
DEX	dexamethasone
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified eagle's media
DMSO	dimethyl sulfoxide
EDTA	ethyline diaminetetra-acetic acid
Egr-1	Early growth response protein 1

EMSA	electrophoretic mobility shift assay
ER	endoplasmic reticulum
ERK	extracellular related kinase
EST	expressed sequence tag
FA	Fanconi's anaemia
FAA/B/C/D/E/F	FA complementation group A/B/C/D/E/F
FCS	fetal calf serum
FSH	follicle stimulating hormone
FSH β	FSH beta subunit
G	guanine
GAP	GnRH associated peptide
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GH	growth hormone
GFP	glass filter paper
GnRH	gonadotrophin releasing hormone
GnRH-R	GnRH receptor
GnRH-RE	GnRH response element
GSE	gonadotrope specific element
GTP	guanosine triphosphate
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethane sulphonic acid]
HPA	hypothalamic pituitary gonadal axis
hpg	hypogonadal mouse
IP ₃	inositol 1,4,5-triphosphate
IP ₃ -R	IP ₃ receptor
IPTG	isopropyl β -D-thiogalactopyranoside
JNK	c-jun N-terminal kinase
Kb	kilobases
KDa	kilodaltons
LB	Luria Bertani
LH	luteinising hormone
LH β	LH beta subunit
Lhx-3	LIM homeobox gene
MAPK	mitogen activated protein kinase
MAPKK	MAPK kinase
MAPKKKK	MAPKKK kinase
MEK	MAPK kinase inhibitor
MLC	myosin light chain
MLCK	MLC kinase
MOPS	3N-Morpholino propanesulfonic acid

MRG1 mRNA	melanocyte specific gene regulated gene 1 messenger RNA
N-terminal NSF	amino terminal N-ethylmaleimide sensitive factor
OD OVX	optical density ovariectomised
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCOS	polycystic ovarian syndrome
PCR	polymerase chain reaction
PGBE	pituitary glycoprotein hormone basal element
PIP2	phosphatidylinositol 4,5-bisphosphate
Pit-1	pituitary factor-1
Pitx1/Pitx2	pituitary homeobox 1/2
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PM	plasma membrane
PMA	phorbol myristate acid
POMC	proopiomelanocortin
Ppi	pyrophosphate
Prop1	prophet of Pit-1
RNA	ribonucleic acid
RNase	ribonuclease
RPA	RNase protection assay
rpm	revolutions per minute
Rpx	Rathke's pouch homeobox
RT-PCR	reverse transcriptase PCR
SDS	sodium dodecyl sulphate
SF-1	steroidogenic factor 1
SgII	secretogranin II
SNAP	soluable NSF attachment protein
SNARE	SNAP receptor
SSCP	single stranded conformational polymorphism
SRE	serum response element
T	thymine
TAE	tris acetate EDTA
TBE	tris borate EDTA
TCA	tri-chloroacetic acid
TCR	transcription coupled repair
TE	tris EDTA

TGN	<i>trans</i> golgi network
TGFβ	transforming growth factor beta
TLC	thin layer chromatography
TPA	12-O-tetradecanoylphorbol-13-acetate
TSH	thyroid stimulating hormone

USF	upstream stimulatory factor
UV	ultra-violet

V	volts
VMH	ventro-medial hypothalamic nucleus

X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
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All DNA sequences are shown in the 5' to 3' orientation. All protein sequences are shown in the amino terminal to carboxy terminal orientation.

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Chapter one: Literature Review

1.1 Introduction

The gonadotrophins, luteinising hormone (LH) and follicle stimulating hormone (FSH), are essential for reproduction. Lack of LH and FSH leads to subfertility in the male and sterility in the female. They are synthesised and secreted from the gonadotroph cells of the anterior pituitary, in response to gonadotrophin releasing hormone (GnRH) released from the hypothalamus. LH and FSH are members of the glycoprotein hormone family. They consist of a common alpha subunit, α glycoprotein subunit (α GSU) and a function specific β subunit; LH β and FSH β , which are differentially expressed in response to GnRH. α GSU and LH β subunit are expressed at a basal level within gonadotroph cells, and are up-regulated by GnRH. However the frequency of GnRH administration is crucial, α GSU is up-regulated in response to constant GnRH administration, but LH β transcription is only up-regulated with a pulsatile administration of GnRH (Mercer and Chin 1995). FSH β transcription is not as strongly affected by GnRH pulsatility, in fact a significant up-regulation of FSH β subunit transcription only occurs in the presence of activin (Turgeon, Kimura et al. 1996; Dalkin, Haisenleder et al. 1999; Graham, Nusser et al. 1999).

Until recently, the focus of study has been on the physiological role of LH and FSH in regulating gonadal function. With the advent of molecular biology and transgenic technology, the differential regulation of LH β , FSH β and α GSU biosynthesis is being unraveled. This literature review aims to describe the current understanding of both basal and GnRH regulated transcription, and the factors which mediate this. An overview of the

physiological roles of LH and FSH and their importance in reproductive function will be followed by a detailed discussion of the development of the pituitary and the transcription factors that are responsible for programming this development and controlling basal gonadotrophin gene expression. The mechanisms that control the differential regulation of gonadotrophin gene expression will be discussed, including the signalling pathways that may be involved in this. The factors involved in up-regulating the gonadotrophin subunits in response to GnRH and the effect of gonadal peptides and steroid hormones will be described. Finally, the regulation and mechanism of secretion of LH and FSH from the gonadotrophs will be discussed.

1.1.1 Hypothalamic pituitary gonadal axis (HPA)

The hypothalamic pituitary gonadal axis (HPA) describes the positive and negative hormonal feedback mechanisms that exist between these three glands. The ultimate driver of the HPA is the hypothalamus, which secretes gonadotrophin releasing hormone (GnRH), a decapeptide of the following amino acids; Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly. This acts on the anterior pituitary, specifically the gonadotroph cells, via the GnRH receptor (GnRH-R). GnRH is released from the hypothalamus in discrete pulses, generally once every 1 or 2 hours, except at the LH surge, and its action results in pulsatile secretion of LH and FSH from the gonadotrophs.

1.1.2a The Female Reproductive Cycle

GnRH, LH, and FSH control the reproductive cycle in non-reflex ovulatory females. Reflex ovulators only ovulate after mating and are not discussed in

this thesis. The reproductive cycle is known as the menstrual cycle in primates, and the oestrus cycle in rodents. It can be divided into two phases, follicular and luteal, which are separated by the LH surge (see Fig. 1.1.2a). The follicular phase is characterised by a steady increase of oestrogen, secreted from developing follicles, whose development is initially dependent on FSH, and low levels of progesterone (see Fig. 1.1.2b). These small follicles also secrete activin and inhibin, which are involved in selecting the dominant follicle (Perheentupa, Critchley et al. 2000). Inhibin also inhibits secretion of FSH, therefore maintaining a low serum level. During this phase, GnRH is secreted approximately every fifteen minutes. As the LH surge approaches, the pulses of GnRH increase in frequency, to the point where they are indistinguishable as individual pulses, and instead are constantly elevated (Clarke, Thomas et al. 1987). The transition to the luteal phase is signalled by a sharp surge in LH secretion (see Fig. 1.1.2c), triggered as a consequence of the increase in GnRH, and concomitant with an increase of oestradiol (Pant, Hopkinson et al. 1977). This in turn inhibits the synthesis of inhibin and activin, which results in an increase in FSH serum levels (McNeilly 1988; Baird, Campbell et al. 1991). The LH surge, triggers release of the ovum from the mature follicle (ovulation). The remaining cells, left behind after the follicle has ruptured, develop into the corpus luteum, which in primates secrete oestradiol and progesterone hence the luteal phase is characterised by a high level of progesterone (Hauger, Karsch et al. 1977). The cells of the corpus luteum also secrete large amounts of inhibin, which is involved

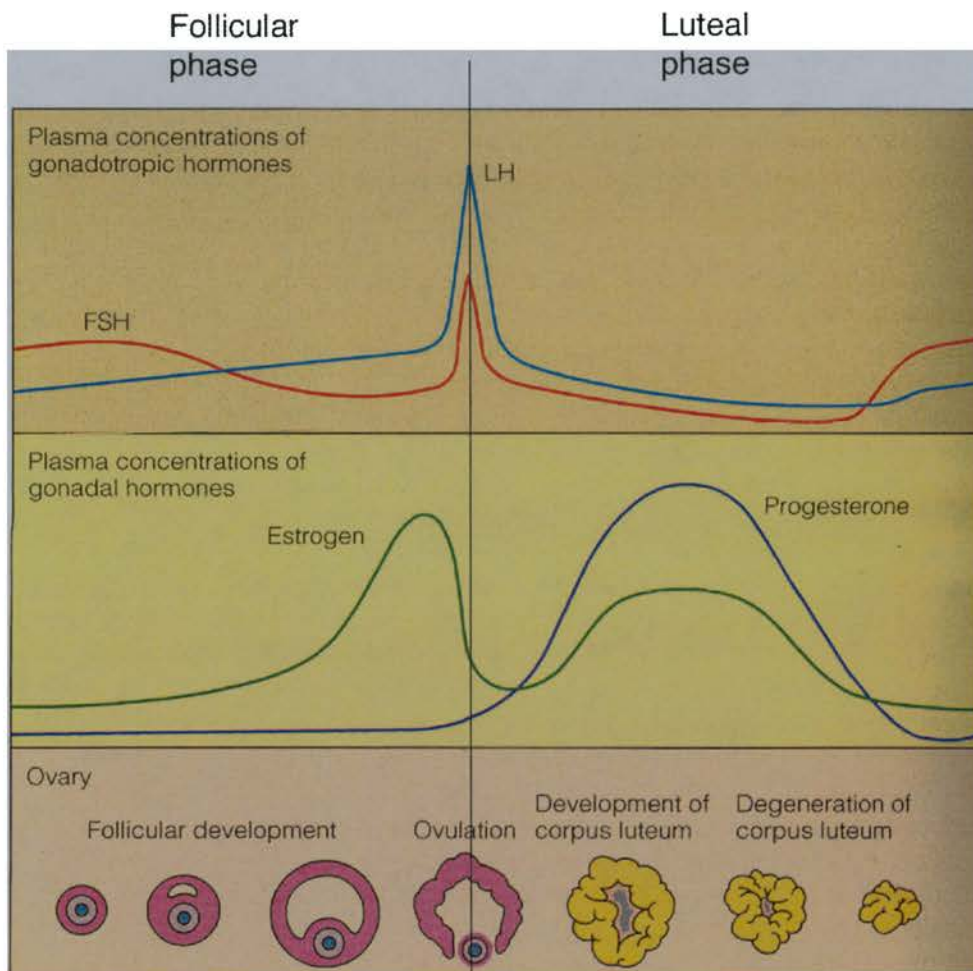


Fig. 1.1.2a: A diagram of the reproductive cycle showing the plasma levels of the gonadotrophins, gonadal hormones, and the follicular development of an ovum in the ovary during the cycle.

This diagram has been adapted from Human Physiology 2nd edition Lauralee Sherwood.

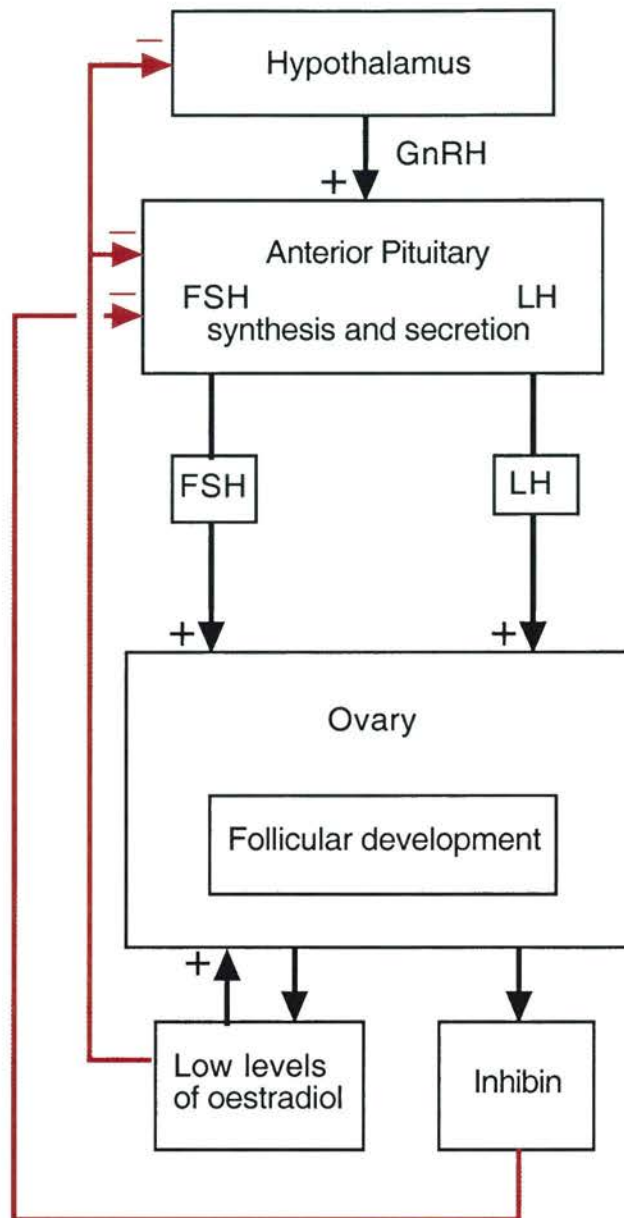


Fig. 1.1.2b: Feedback control of FSH and LH secretion during the Follicular Phase.

Adapted from Human Physiology, Lauralee Sherwood.

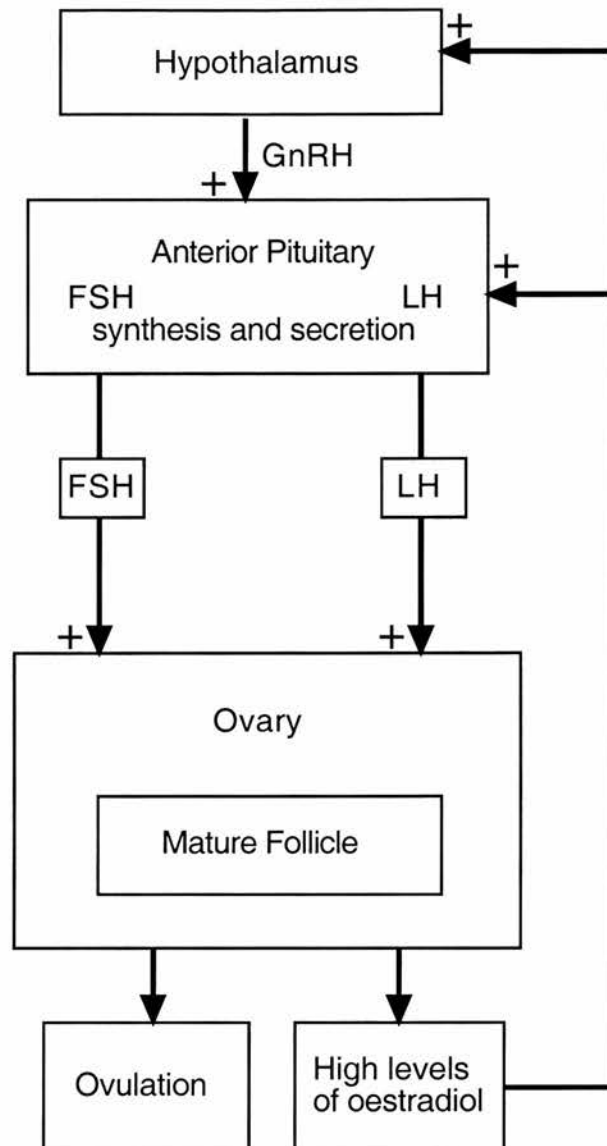


Fig. 1.1.2c: Control of the LH surge at ovulation.
Adapted from Human Physiology 2nd Ed. Lauralee Sherwood.

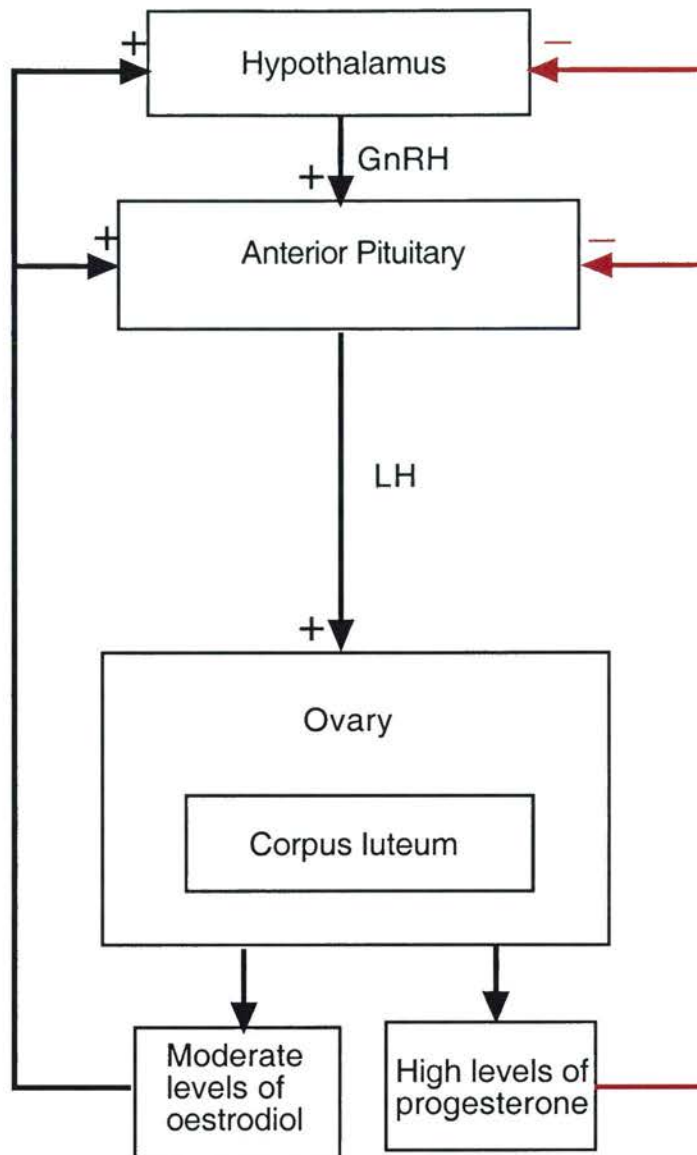


Fig. 1.1.2d: Feedback control during the Luteal Phase.
Adapted from Human Physiology 2nd Ed. Laurelee Sherwood.

in maintaining the low level of FSH during the luteal phase (see Fig. 1.1.2d). If the ovum is not fertilised and implanted, then the corpus luteum is broken down, the levels of progesterone decrease, and the cycle enters the follicular phase once more.

1.1.2b The Rodent Oestrus Cycle

The rodent oestrus cycle is divided into 4 stages, oestrus, met-oestrus, di-oestrus and pro-oestrus. The oestrus cycle of rodents is similar to the menstrual cycle of primates, but there are some differences (Greenwald and Roy 1994). In rodents, there is a high level of FSH due to reduced circulating levels of inhibin, which produces relatively large follicles. Therefore the follicles require a shorter period of growth and hence the follicular phase (proestrus) is short, lasting for 1 to 2 days (Greenwald and Choudary 1969). If the ovum remains unfertilised, the corpus luteum degenerates within 2 to 3 days (Marshall 1984). The rapid degeneration of the corpus luteum leads to a shorter luteal phase and contributes to the decreased levels of circulating inhibin (Marshall 1984).

1.1.3 The Male

FSH and LH control spermatogenesis and testosterone secretion respectively. LH acts on the Leydig cells, stimulating production of testosterone, which is involved in mitosis and meiosis of the germ cells, and FSH acts on the seminiferous tubules, specifically the Sertoli cells, to enhance spermatogenesis, and spermatid remodelling (Sherwood 1993). Again, as in the female, there are negative feedback loops that act on both LH and

FSH (see Fig. 1.1.3). Testosterone exerts most of its inhibitory effects on LH, by inhibiting the pulse frequency of GnRH at the level of the hypothalamus and decreasing the GnRH receptor sensitivity to GnRH at the anterior pituitary. Inhibin is also secreted from the Sertoli cells and negatively regulates FSH β synthesis and secretion (Carroll, Corrigan et al. 1989).

1.1.4 Disruption of GnRH, LH or FSH causes gonadal abnormalities and can lead to infertility.

The importance of regulating gonadotrophin synthesis and secretion has been shown in a number of mouse models, and in humans with mutations in gonadotrophin genes that result in subfertility, or infertility. Transgenic mice models in particular, have helped to define the reproductive role of each gonadotrophin subunit.

A transgenic mouse with a targeted disruption of α GSU was reported in 1995 (Kendall, Samuelson et al. 1995). Since α GSU dimerises with LH β , FSH β and thyroid stimulating hormone β (TSH β) to form functional hormones, the mice lacked TSH, LH and FSH. These mice displayed hypogonadism and also hypothyroidism, which resulted in dwarfism. Thyroid development was arrested in late gestation suggesting that TSH was required for the development of the thyroid. However foetal and neonatal gonadal development were normal, so gonadotrophins are only required for gonadal maturation and genital development after birth, and they are not required for sexual differentiation, or genital development in the foetal rodent. So, the reproductive role of the gonadotrophins has been defined in a number of other studies that report patients with mutations in LH β , FSH β

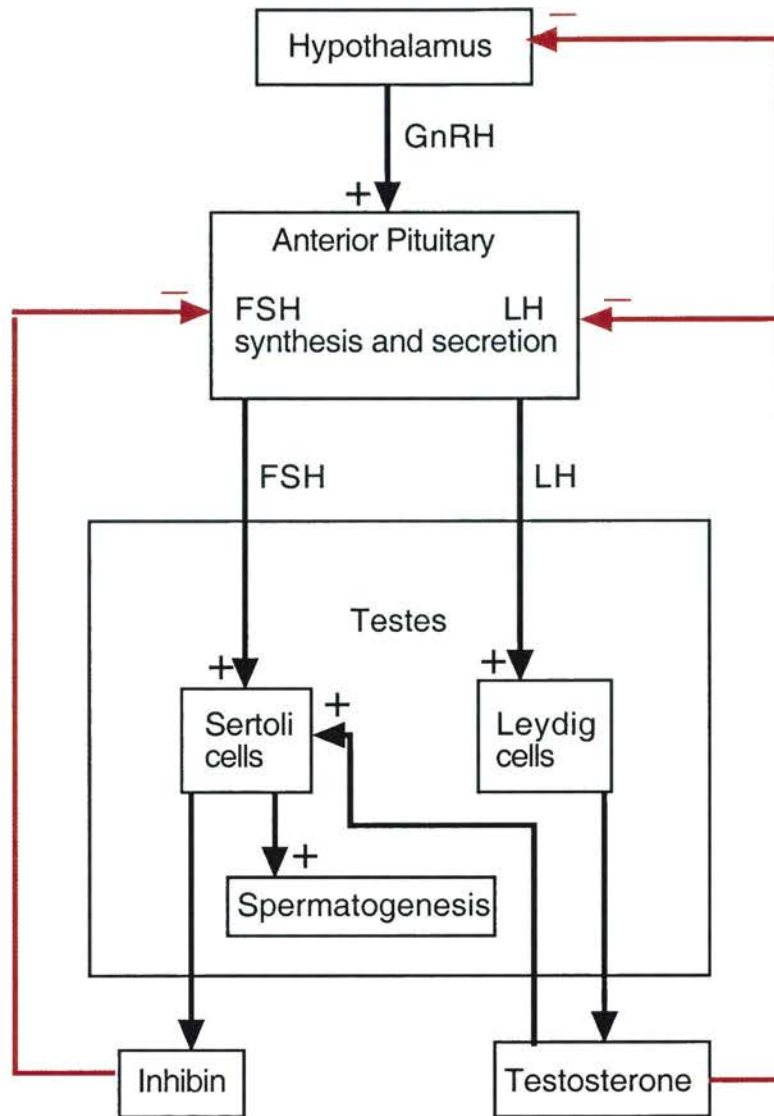


Fig.1.1.3: Control of Testicular Function.
Adapted from Human Physiology 2nd Ed. Lauralee Sherwood.

and the LH and FSH receptors. Additionally, transgenic mice have also been used to confirm the role of the individual gonadotrophin subunits, these studies are described below.

A frameshift deletion in the *FSH β* gene of a female patient resulted in FSH deficiency, with amenorrhoea, infertility, and an elevated LH circulating plasma level (Matthews, Borgato et al. 1993). An inactivating mutation in the FSH receptor in men essentially leading to lack of FSH function, resulted in subfertility and reduced spermatogenesis (Tapanainen, Aittomaki et al. 1997). To study the role of FSH in fertility, a *FSH β* gene disrupted mouse was generated (Kumar, Wang et al. 1997). The testes of these mice were smaller than their wild type littermates, and histological analysis showed a decrease in the number of Sertoli cells, however the stages of spermatogenesis appeared normal and the seminiferous tubules contained mature spermatozoa. This is in keeping with the reported role of FSH in Sertoli cell multiplication during embryonic development (Kluin, Kramer et al. 1984; Orth 1984; Orth 1986). The ovaries and uteri of the female mutants were also examined, and were shown to be smaller and thinner than their wildtype counterparts, moreover, folliculogenesis was abnormal. The ovaries of the mutant mice lacked any corpora lutea or normal follicles beyond the primary follicle stage, suggesting that these mice did not undergo normal oestrus cycles and infertility of the female mice was due to a block in follicle maturation. Examination of the serum LH levels of these mice revealed that LH levels were only elevated in the female mice, therefore the mutant female mice mimicked the female patient with primary amenorrhoea described previously. Genetic rescue of these gene disrupted mice was performed by targeting expression of a human *FSH β* transgene to the

pituitary, this rescued the phenotype of the mutant mice and the females showed normal fertility (Kumar, Low et al. 1998). FSH receptor gene disrupted mice have a similar phenotype to *FSH β* gene disrupted mice. However, the female transgenic mice had high serum levels of FSH and LH, which may be due to a loss of oestradiol negative feedback from the ovaries. The male mice were fertile, which further suggests that testosterone, not FSH is required for spermatogenesis (Abel, Wootton et al. 2000). Recently, a transgenic mouse model with intact FSH synthesis and secretion, but lacking LH synthesis and secretion was described (Allan, Haywood et al. 2001). This model was used to identify the *in vivo* effects of FSH alone. In the male transgenic mouse, FSH appears to induce the completion of meiosis, however further progress in spermatogenesis does not occur. FSH is not involved in Leydig cell development or function, but does appear to affect Sertoli cell function. The ovaries of the transgenic mice were larger than the control mice, and showed increased follicle recruitment and development, however there was no evidence of corpus luteum formation or oestradiol response to FSH.

A *LH β* gene disrupted mouse has not yet been described, so the *in vivo* role of LH has been elucidated mainly by over expression models, however a LH receptor/choriogonadotrophin (CG) receptor knockout has recently been reported (Lei, Mishra et al. 2001). These mice had under-developed gonads, the null males displaying micropenes, Leydig cell disruption, seminiferous tubule disarray, and arrested spermatogenesis. The female null mice displayed under-developed uteri and ovaries with arrested follicular growth. Both mice had elevated serum LH levels, but low testosterone, oestradiol and progesterone levels, and both male and female mice were infertile.

Patients with a deactivating mutation in the LH receptor have also been reported and present with reproductive abnormalities. Males have been reported with pseudohermaphroditism, displaying female genitalia, and abdominal testes with fully differentiated Sertoli cells, but an absence of Leydig cells (Berthezene, Forest et al. 1976). A female carrying this mutation presented with a milder phenotype, displaying primary amenorrhoea, with both primary and secondary sexual characteristics (Toledo, Brunner et al. 1996). The ovaries of this female displayed all stages of follicular development, however there was an absence of preovulatory follicles and corpora lutea. Both male and female patients show elevated serum LH levels, and low levels of testosterone, oestradiol, and progesterone. The male showed normal FSH levels, but the female showed elevated FSH levels (Themmen and Huhtaniemi 2000).

Female patients that have elevated serum androgen levels present with hirsutism, chronic anovulation, polycystic ovaries and infertility, otherwise described as polycystic ovary syndrome (PCOS) (Franks 1989). These patients also present with elevated levels of LH. A transgenic mouse that over-expressed LH was developed in 1996, and appeared to share some of the phenotypic symptoms of PCOS patients. The female mice exhibited enlarged ovaries with multiple corpora lutea, ovarian cysts and tumours and were anovulatory (Risma, Clay et al. 1995). Further analysis of these mice revealed that elevated LH caused precocious puberty (Risma, Hirshfield et al. 1997), consistent with reports of a male patient with an activating LH receptor mutation (Schedewie, Reiter et al. 1981; Rosenthal, Grumbach et al. 1983), and hyperandrogenemia consistent with PCOS (Risma, Hirshfield et al. 1997).

The report of a mutant mouse with hypogonadism (Cattanach, Iddon et al. 1977) has allowed further studies of the control of synthesis and secretion of the gonadotrophins. Hypogonadal mice (hpg) have immature gonads and the males presented with small penes, an under-developed scrotum, and small testes. Females present with failure of the vagina to open, an under-developed uterus and very small ovaries (Cattanach, Iddon et al. 1977). Concentrations of LH and FSH levels in the pituitary were markedly reduced compared with normal mice, and GnRH levels were undetectable (Cattanach, Iddon et al. 1977). Thus the hypogonadism seen in these mice is due to a loss of GnRH stimulation on the gonadotrophs. Plasma LH could be restored with administration of GnRH, but not with stimulation of the hypothalamus, implying that the loss of GnRH is due to a loss of synthesis at the hypothalamic level, rather than a loss of sensitivity to GnRH at the pituitary level (Charlton, Halpin et al. 1983; Lewis, Morris et al. 1986). The mutation in these mice has been localised to a partial deletion in the *GnRH* gene (Seeburg, Mason et al. 1989). This deletion removes two exons that encode the GnRH associated peptide (GAP) moiety of the GnRH mRNA precursor, resulting in a nonfunctional mRNA GnRH precursor, and hence no GnRH peptide. In the males, some testosterone synthesis occurred indicating that the gonadotrophs were intact and capable of synthesising some LH (Fink, Sheward et al. 1984).

Therefore, GnRH is required to up-regulate gonadotrophin levels. FSH is required for follicle recruitment in females and Sertoli cell function and possibly development in males. LH appears to be directly involved in ovulation, and indirectly involved in spermatogenesis. Loss of both hormones results in hypogonadism.

1.2 Development of the Anterior Pituitary

1.2.1 The initial patterning of the anterior pituitary.

The anterior pituitary consists of 5 different cell lineages, characterized by the hormones they produce. These are; the adrenocorticotropin (ACTH) secreting corticotrophs, thyrotrophs, which secrete thyroid stimulating hormone (TSH), somatotrophs that secrete growth hormone (GH), lactotrophs which secrete prolactin, and the gonadotrophs, which secrete LH and FSH (Japon, Rubinstein et al. 1994; Dasen and Rosenfeld 2001).

Differentiation of the pituitary cell types depends on a signalling gradient similar to initial patterning in drosophila (Treier and Rosenfeld 1996). Pituitary factor 1 (Pit1) and a zinc finger transcription factor (GATA2), that binds the DNA sequence (A/T)GATA(A/G), are required and sufficient to determine the terminal differentiation of all the anterior pituitary cell types except the corticotrophs (Simmons, Voss et al. 1990). Initially, the ventral dorsal bone morphogenic protein 2 (BMP2) gradient induces GATA2 in a corresponding gradient in the developing thyrotrophs and gonadotrophs (Couly and Le Douarin 1988; Ericson, Norlin et al. 1998). The high levels of GATA2 restrict the expression of Pit1 in the forming gonadotrophs. At a more dorsal region, the level of GATA2 decreases, and the inhibition of Pit1 expression is removed. Cells in this region will express both GATA2 and Pit1, and will develop into thyrotrophs. The most dorsal cells express high levels of Pit1, which inhibit the effects of GATA2. The cells in this region will go on to develop into somatotrophs and lactotrophs. Mutating the homeodomain of either transcription factor disrupts this gradient and leads to a loss of one or more cell types (Dasen, O'Connell et al. 1999). This

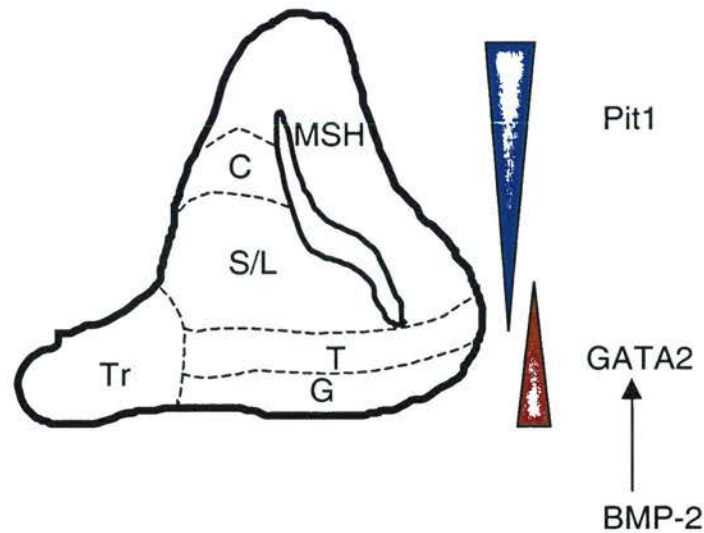


Fig. 1.2.1: A diagram showing the cells of the pituitary and the signalling gradient of transcription factors required to organise the spatial arrangement of the pituitary.

BMP-2 is initially expressed at the border between Rathke's pouch and the oral ectoderm at embryonic day 10.5 (e10.5) and establishes a ventralsdorsal signalling gradient and initiates GATA2 expression in a corresponding gradient (red arrow). Pit1 expression is detected at e13.5, however its expression is restricted by high levels of GATA2 (expression level is indicated by the blue arrow) therefore, cells that express Pit1 alone form the somatotrophs, lactotrophs (S/L) and melanotrophs (MSH) and cells that express Pit1 and GATA2 form the thyrotrophs (T). Cells that express GATA2 alone form the gonadotrophs (G). The rostral tip thyrotrophs are also indicated (Tr).

(Adapted from Dasen, J. and Rosenfeld, M.J. 1999).

overlapping gradient is required for the correct temporal transcriptional activation of factors that positionally determine the different cell-types (see Fig. 1.2.1). The gradient also delineates the mature differentiated cell, by activating expression of transcription factors that specifically regulate expression of the hormonal markers that identify the cell-type.

1.2.2 Specification of basal transcription factor gene expression and how these regulate expression of gonadotrophin subunit genes

Transcription of the gonadotrophin subunits is highly regulated, and can be divided into basal regulated transcription (non-GnRH), and GnRH stimulated transcription. In this section I will discuss the known transcription factors, which are involved in specifying the pituitary and transcribing the gonadotrophin subunits, and I will discuss the factors, which control GnRH regulated gonadotrophin subunit transcription, in a later section.

1.2.2a Rpx/Hesx1

One of the first transcription factors activated in the pituitary primordium is Rathke's pouch homeobox (*Rpx*), which can be detected as early as embryonic day 6.5-7 (e6.5-7) in the anterior neural plate of the embryo (Thomas, Johnson et al. 1995). Its expression however is quickly restricted to Rathke's pouch and is extinguished by e13.5 with the activation of the pituitary specific POU domain transcription factor Pit-1 (Hermesz, Mackem et al. 1996). The role of *Rpx* appears to be in the initial determination of the anterior (prechordal) region of the embryo, and in restricting the further differentiation of the anterior pituitary. Analysis of targeted *Rpx* gene

disrupted mice show that *Rpx* is essential for the development of the forebrain, eyes, and other anterior structures, including development of the anterior pituitary. Furthermore, the associated mutation in man causes combined pituitary hormone deficiency (CPHD) (Dattani, Martinez-Barbera et al. 1998).

1.2.2b LIM proteins

The next step in the development of the anterior pituitary is the differentiation of the different cell types. The LIM homeobox gene *P-Lim* or *Lhx-3* (Bach, Rhodes et al. 1995), is expressed throughout development in the pituitary and is also expressed in the adult. Histological analysis of *Lhx-3* gene disrupted mice showed that these mice lacked the anterior and intermediate lobes of the pituitary (Sheng, Zhadanov et al. 1996). Growth arrest was also accompanied by changes in the expression of the pituitary specific marker genes. In the wild type mouse, *Rpx* expression is still detected at e12.5, along with *Lhx-3* expression, indicating that initial expression of *Rpx* is independent of *Lhx-3* protein. However, in the null mutant *Rpx* expression had ceased by e12.5. α GSU, which is expressed in thyrotropes and gonadotrophs, is the earliest pituitary specific marker and is missing in *Lhx-3* mutants (Simmons, Voss et al. 1990). Thus *Lhx-3* is essential for the differentiation and proliferation of the gonadotroph, thyrotroph, somatotroph and lactotroph cell lineages, and is also required for the continued expression of *Rpx* and transactivation of α GSU (Bach, Rhodes et al. 1995). A further LIM protein, *Lhx-2* can also bind and stimulate the α GSU promoter (Roberson, Schoderbek et al. 1994) and is required for basal expression of α GSU *in vitro*. Recently a melanocyte specific gene-regulated gene 1 (MRG1) has been identified by yeast two hybrid analysis, to interact

with *Lhx-2*. *MRG1* can trans-activate *α GSU* gene expression, and mutation of the *Lhx-2* DNA binding site disrupted this activation (Glenn and Maurer 1999).

1.2.2c *Pitx1*

In the *Lhx-3* mutant, all but the corticotrophs fail to form in the anterior pituitary. Therefore, another factor induces corticotroph development. The homeobox transcription factor, pituitary homeobox factor-1 (*Pitx1*), was isolated as a transcription factor involved in control of proopiomelanocortin (*POMC*) gene expression (Lamonerie, Tremblay et al. 1996). It is expressed throughout development of the pituitary, and is the earliest known marker for all stomodeal derivatives including Rathke's pouch (Lancot, Lamolet et al. 1997; Lancot, Moreau et al. 1999). However it is also a potent activator of several pituitary specific promoters and interacts with lineage restricted transcription factors to achieve cell and promoter specific transcriptional activation (Tremblay, Goodyer et al. 2000). On prolactin and growth hormone promoters it interacts with *Pit1* (Tremblay, Lancot et al. 1998; Szeto, Rodriguez-Esteban et al. 1999), with *NeuroD1/Pan1* on the *POMC* promoter (Poulin, Turgeon et al. 1997), and with steroidogenic factor-1 (*SF-1*) and early growth factor protein-1 (*Egr-1*) on the *LH β* promoter (Tremblay, Marcil et al. 1999). *Pitx1* gene deleted mice however, still have an intact pituitary, indicating that it does not have a role in pituitary development (Szeto, Rodriguez-Esteban et al. 1999) and suggests that there may be functional redundancy between this factor and another paired-like homeobox transcription factor *Pitx2*. Disruption of *Pitx2* causes Reiger syndrome (Lu, Pressman et al. 1999), but the function of this factor in regulating pituitary gene expression has not been investigated.

Pitx1 activates the LH β , FSH β and α GSU promoters, and deletion of the Pitx1 binding site, disrupts this activation. In addition, Pitx1 also acts in synergy with SF-1 to up-regulate LH β promoter activity (Tremblay, Lanctot et al. 1998) by a protein-protein interaction between the C-terminus of Pitx1 and the N-terminal half of SF-1 (Tremblay, Marcil et al. 1999). It is also essential for α GSU and *Lhx3* gene expression, the mRNA levels of both are virtually undetectable in α T3-1 cells that have been transfected with an anti-sense Pitx1 clone (Tremblay, Lanctot et al. 1998). The Pitx1 and SF-1 binding sites are 20bp apart on the LH β promoter, and are conserved in many species, both can independently activate transcription of the LH β promoter but they can also act in synergy, to specifically up-regulate LH β transcription (Halvorson, Kaiser et al. 1996; Tremblay, Marcil et al. 1999).

1.2.2d Pit1

As development progresses two other transcription factors appear, the POU domain transcription factor, Pit1 and the orphan nuclear receptor, SF-1. Both are first detectable at e13.5 in the anterior pituitary (Treier and Rosenfeld 1996).

Pit1 is a member of the POU family of transcription factors and regulates the genes encoding growth hormone (GH), prolactin and TSH β subunit. During the development of the anterior pituitary Pit1 expression is switched on as Rpx expression is switched off. Mutations in either *Pit1* (Snell dwarf mutants) or prophet of *Pit1* (*Prop1*) / Ames dwarf gene (*df*) (Sornson, Wu et al. 1996) lead to severe proportional dwarfism, hypothyroidism, and infertility (Radovick, Nations et al. 1992). *Prop1* null mice have anterior pituitary

hypocellularity, due to a reduction in thyrotropes, somatotropes and lactotropes (Gage, Brinkmeier et al. 1996). Two complete loss of function alleles of *Pit1* leads to the failure to activate *TSH β* , GH and prolactin, and loss of cell proliferation after e14.5 (Li, Crenshaw et al. 1990). This evidence suggests that *Pit1* and *Prop1* lie on a common development pathway, which involve the formation of the thyrotropes, somatotropes and lactotropes. *In situ* hybridisation has shown that *Prop1* is required for the repression of *Rpx*, long before initial activation of *Pit1* and indeed may be responsible for the activation of *Pit1* by day e15.5 (Gage, Brinkmeier et al. 1996).

It has also been shown that *Pit1* synergises with P-LIM and may be involved in the activation of *TSH β* and prolactin genes, though P-LIM is not required for *Pit 1* activation (Bach, Carriere et al. 1997).

1.2.2e Steroidogenic factor 1 (SF-1)

SF-1 is classified as an orphan nuclear receptor, since an activating ligand has not yet been identified (Duval, Ellsworth et al. 1999). Its importance in endocrine function was first illustrated in *SF-1* gene deleted mice, equivalent to *Ftz-F1* gene deletions in *Drosophila* (Luo, Ikeda et al. 1994). The null mice, all died within eight days, and when analyzed for corticosterone levels, were found to have lower levels than normal. This indicates that death was due to adrenocortical insufficiency. These mice were also devoid of adrenal glands and gonads, unsurprising since the gonads are the second major site of SF-1 expression in embryogenesis (Ikeda 1996). Therefore, SF-1 is essential for gonadal development. SF-1 also acts at the pituitary, immunohistochemical analysis revealed that these *SF-1* gene disrupted mice were also lacking the gonadotrope specific markers, LH β and FSH β , and pituitary expression of

GnRH-R transcripts was also undetectable. However TSH, GH, ACTH were present (Ingraham, Lala et al. 1994).

Further studies showed that SF-1 was also expressed in the ventral diencephalon, which gives rise to the endocrine hypothalamus, suggesting that it may act at other levels of the reproductive axis. Immunohistochemical analysis in the adult mouse brain revealed that expression of SF-1 was restricted to the ventral medial hypothalamic nucleus (VMH). In *SF-1* gene disrupted mice the VMH is structurally abnormal, with decreased cellularity and organisation of the nucleus. In developing embryos, the gene-disrupted mice had early stages of VMH development, which later regressed.

The *SF-1* gene disrupted mice were treated with GnRH, and the gonadotrophin expression profile was restored. This indicated that the gonadotrope lineage was not ablated, in fact the mice were able to synthesise GnRH however they were unable to secrete it (Ikeda, Luo et al. 1995). Recently, a pituitary specific *SF-1* gene disrupted mouse has been described (Zhao, Bakke et al. 2001). This transgenic mouse was viable but infertile, and the gonads were hypoplastic. The LH and FSH expression levels in these mice were also decreased, suggesting a pivotal role for SF-1 in gonadotrophin expression (Bakke, Zhao et al. 2001; Zhao, Bakke et al. 2001).

SF-1 DNA binding sites are known as gonadotrope specific elements (GSE) and are found in the promoter regions of both LH β subunit (Brown, McNeilly et al. 1993; Keri and Nilson 1996) and α GSU (Horn, Windle et al. 1992; Barnhart and Mellon 1994). Gel electrophoretic mobility shift assays (EMSA) determined that SF-1 bound to the GSE, and that mutagenesis of the DNA sequence, identified a consensus SF-1 DNA binding site TGACCTTGTT (Halvorson, Kaiser et al. 1996). Transfection studies showed that SF-1

transactivates both α GSU (Barnhart and Mellon 1994), and rat LH β promoters (Halvorson, Kaiser et al. 1996) *in vitro* and the cow LH β promoter in transgenic mice (Keri and Nilson 1996). SF-1 transcription has been reported to be up-regulated by GnRH administration (Haisenleder, Yasin et al. 1996) however, other reports have shown that SF-1 transcription is not affected by GnRH *in vivo* (Brown and McNeilly 1997). SF-1 therefore poses a conundrum, it is clearly an essential co-factor in up-regulating transcription, but numerous studies have shown that its mRNA levels are unvarying (Parker and Schimmer 1997), although transcriptional up-regulation of the target gene is seen in response to peptide hormone action. This would suggest that SF-1 activation is brought about by post-translational modifications, i.e. phosphorylation (Zhang and Mellon 1996). This has been located to a single serine residue (Ser-203) within the activation domain of SF-1 (Hammer and Ingraham 1999).

Drean (Le Drean, Liu et al. 1997) was able to show that SF-1 could recruit different transcription factors, depending on distinct *cis* acting elements on gonadotropin gene promoters. Therefore, SF-1's role in regulating transcription may be to recruit specific transcription factors to specific promoters. It also regulates gonadotrope gene transcription by synergising with other factors.

1.3 GnRH Regulation of Gonadotrophin Subunit Expression

The gonadotrophs are unique in the anterior pituitary in that they transcribe, translate, and secrete LH and FSH differentially in response to GnRH. This means that GnRH can act at different sites after receptor activation. The signalling pathways from the receptor to either the nucleus, or the secretory mechanisms are not characterised. However, a number of different mechanisms have been postulated for controlling this, which include; pulse frequency, pulse amplitude, second messenger pathways, and/or GnRH-R number and turnover. Therefore the available scientific information that starts to unravel how the GnRH signal may be differentially transduced is described below.

1.3.1 *In vivo* studies of GnRH regulation of gonadotrophin synthesis and secretion

Initial studies were carried out in ovariectomised (OVX), and castrated sheep and rats, to remove steroidal and peptide feedback from the gonads. The serum LH and FSH levels in these animals increased, however administration of oestradiol reduced them to pre-gonadectomy levels (Gharib, Wierman et al. 1990), highlighting the significant effect of steroid hormones on synthesis and secretion. However, disrupting the GnRH stimulus to the gonadotrophs by surgically isolating the pituitary from the hypothalamus in OVX ewes, reduced LH and FSH levels, which demonstrated the importance of pulsatile administration of GnRH.

Interestingly, manipulation of GnRH pulse frequency in the hypogonadal mouse, substantiated that synthesis and secretion of LH and FSH was sensitive to the pattern of GnRH administration. Administration of a single

daily injection of GnRH resulted in an increase of serum FSH, and only a slight increase in serum LH levels. However, 12 injections a day increased serum LH and FSH levels to almost normal. The development of the gonads of both male and female mice was increased (the testes and uterine weight increased) suggesting that a more frequent GnRH injection regime, would mimic the condition found in normal mice (Charlton, Halpin et al. 1983).

1.3.2 GnRH pulse Frequency/Amplitude

Before puberty, GnRH is released sporadically from the hypothalamus, and LH and FSH are at basal plasma levels. At puberty, in males, GnRH pulse frequency increases to approximately one an hour and remains at this frequency throughout adult life (Lincoln, Fraser et al. 1985). In contrast, in the adult female, GnRH pulse frequency varies depending on the stage of the reproductive cycle (Knobil 1980). The period that leads up to the LH surge, and triggers ovulation is characterised by an increase in GnRH pulse frequency, initially one every 45-60 minutes, increasing until the GnRH pulses appear continuous, returning to one pulse every hour, after ovulation (Clarke, Thomas et al. 1987; Moenter, Caraty et al. 1991). The pulsatile nature of the GnRH delivery is crucial since a constant infusion of GnRH to either ovariectomised or hypogonadal sheep leads to cessation of LH secretion, with LH β and FSH β mRNA levels dropping to 50% of the levels found in sheep treated with pulses (Mercer, Clements et al. 1989).

The importance of GnRH pulse frequency and amplitude was recapitulated in mice and rats. Experiments carried out in rat models showed that administration of pulsatile GnRH increased mRNA levels of α GSU and LH β

(Haisenleder, Khoury et al. 1987; Haisenleder, Dalkin et al. 1991; Jakubowiak, Janecki et al. 1991). However, the pattern of increase was different, an increase in mRNA levels of LH β occurs with 30 min pulse frequencies (Kaiser, Conn et al. 1997), and LH β mRNA up-regulation is only seen with a specific pulse frequency (Papavasiliou, Zmeili et al. 1986; Shupnik 1990; Weiss, Duca et al. 1990; Haisenleder, Dalkin et al. 1991), whereas α GSU mRNA up-regulation is seen with either continuous or pulsatile GnRH administration.

A more recent study investigated the effect of pulse amplitude on all three subunits. LH β subunit expression was increased in response to low levels of GnRH (35pg/ml) and FSH β and α GSU responses were maximal at 70pg/ml. Interestingly LH release was maintained over a 10 fold range of GnRH pulse dose, suggesting that LH secretion was not coupled to synthesis (Salton, Blum et al. 1988; Weiss, Jameson et al. 1990; Weiss, Crowley et al. 1992; Haisenleder, Ortolano et al. 1993). Therefore GnRH amplitude controls gonadotrophin secretion rather than synthesis, since LH secretion is increased over a broad range of GnRH amplitude administration.

In contrast to α GSU and LH β , FSH β was not significantly up-regulated by either continuous or pulsatile administration of GnRH, in fact only slow GnRH pulse frequencies (one every 120 minutes) increased FSH mRNA levels (Dalkin, Haisenleder et al. 1989). FSH β up-regulation also requires a further signal, which has been identified as activin (Weiss, Crowley et al. 1993; Dalkin, Haisenleder et al. 1996; Graham, Nusser et al. 1999).

1.7.1 GnRH and The GnRH-R

The GnRH signal activates the GnRH-R, which transduces the signal to the nucleus and the secretory machinery through second messenger signalling pathways. There have been reports of a number of structural variants of GnRH (King and Millar 1979) and so far, 14 novel peptides have been identified (Sherwood, Lovejoy et al. 1993; Sealfon, Weinstein et al. 1997). In addition to the classical GnRH peptide another, designated as GnRH II, is present in individual species of most vertebrates studied (Sherwood, Lovejoy et al. 1993; Sealfon, Weinstein et al. 1997; White, Eisen et al. 1998). Recently a GnRH-R that specifically binds this GnRH II has been cloned and characterised (Millar, Lowe et al. 2001), therefore this section will discuss both the GnRH type I receptor, and the GnRH type II receptor.

1.3.3a The GnRH Type I receptor

Type I GnRH (GnRH) binds the GnRH type I receptor (GnRH-R). The gene for the pituitary GnRH-R was originally cloned from α T3-1 cells and encodes a protein of 327 residues (Tsutsumi, Zhou et al. 1992). In addition, the human, rat and sheep genes have also been cloned (Kaiser, Zhao et al. 1992; Chi, Zhou et al. 1993; Illing, Jacobs et al. 1993). Like other G-protein coupled receptors, the GnRH-R has seven transmembrane domains however, the mammalian GnRH-R lacks an intracellular carboxyl terminal tail (see Fig. 1.3.3) (Kakar, Musgrove et al. 1992; Chi, Zhou et al. 1993). Functional and regulatory studies have shown that, unlike other G-protein coupled receptors, the mammalian GnRH-R does not undergo rapid desensitisation (Davidson, Wakefield et al. 1994; Anderson, McGregor et al. 1995; McArdle, Willars et al. 1996) and this was due to the lack of the carboxyl tail

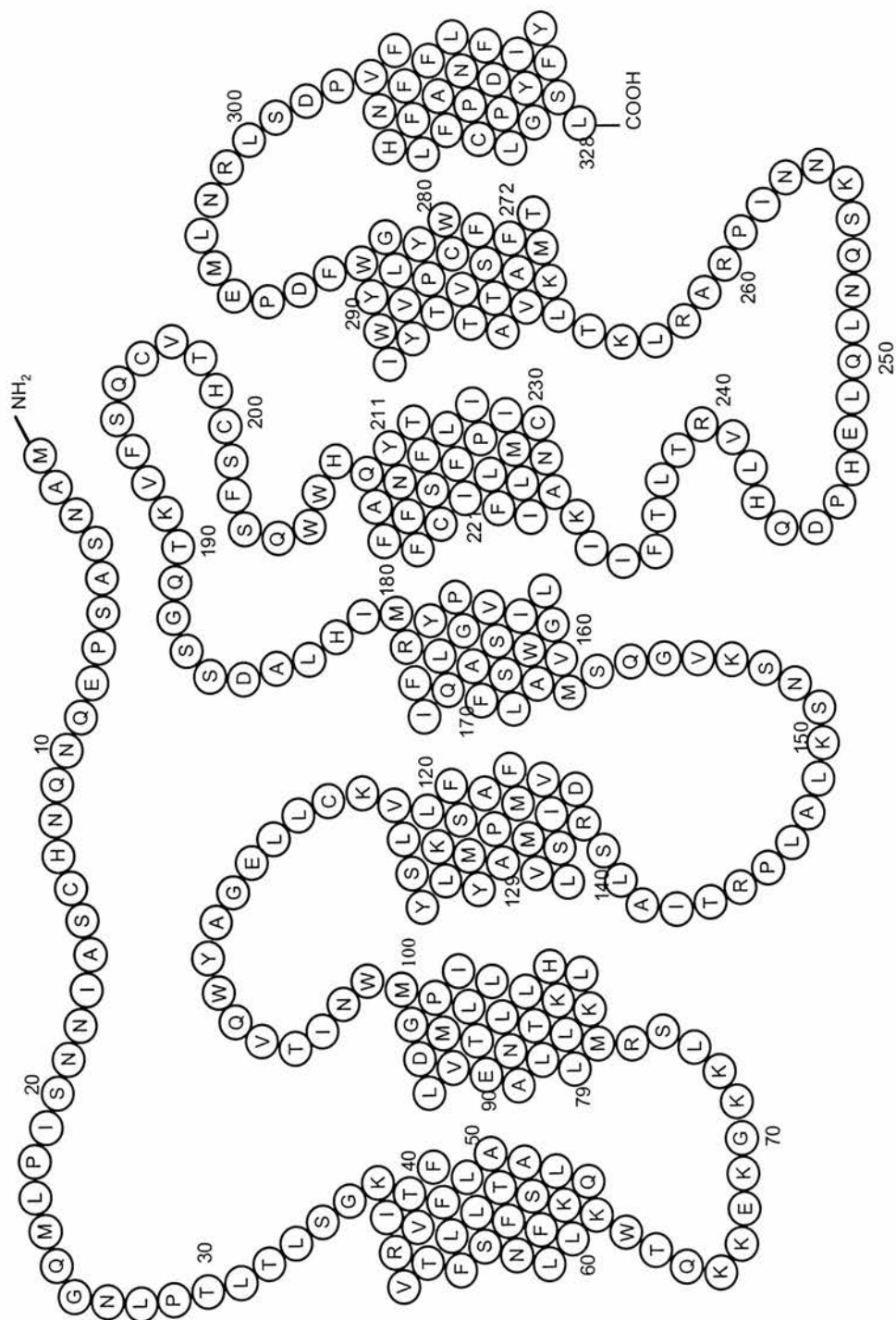


Fig 1.3.3: The GnRH-R (kind gift from Dr T. Ott).

(Willars, Heding et al. 1999; Heding, Vrecl et al. 2000). This means that the GnRH-R is maintained in an agonist sensitive state, which may have implications for the differential regulation of the gonadotrophin subunits.

1.3.3b GnRH II and the GnRH type II receptor

GnRH II is widely distributed in tissues, and has been shown to regulate potassium channels in sympathetic ganglia and stimulate reproductive behaviour (King and Millar 1979; Sherwood, Lovejoy et al. 1993; Maney, Richardson et al. 1997). Other studies have shown that GnRH II can also preferentially stimulate FSH release (Yu, Karanth et al. 1997; Yu, Karanth et al. 2000). Recently the GnRH type II receptor (GnRH-R II) has been cloned from marmoset (Millar, Lowe et al. 2001). It is also a seven transmembrane receptor, however it contains a carboxyl terminal tail. Like the GnRH-R type I, it activates $G_{q/11}$ and extracellular regulated kinase 1/2 (ERK1/2), however it differs in its activation of p38 mitogen activated kinase. It is distributed throughout the brain, especially in areas that are involved in sexual arousal, and in gonadotrophs. Its function in the gonadotrophs has not been fully elucidated, therefore, in this thesis, only type I GnRH and type I GnRH-R are discussed.

1.3.4 GnRH receptor number.

The ability of the gonadotroph cells to respond to GnRH depends on receptor number and turnover. It has been established that GnRH-R numbers vary during the oestrus cycle (Savoy-Moore, Schwartz et al. 1980; Barkan, Regiani et al. 1983) increasing during the period leading to the LH surge (Brooks, Taylor et al. 1993). Furthermore, GnRH-R numbers are

increased by pulsatile administration of GnRH (Katt, Duncan et al. 1985), and GnRH-R gene expression levels are augmented by oestradiol (Yasin, Dalkin et al. 1995). Interestingly, the GnRH-R mRNA levels in female rats appear to be more dependent on GnRH pulse frequency, than in male rats (Yasin, Dalkin et al. 1995), which suggests that GnRH-R number may be involved in controlling the events, such as the LH surge, during the oestrus cycle. GnRH-R mRNA levels also increase in ovariectomised and castrated rats, suggesting that gene expression is regulated by factors that are involved in regulating gonadotrophin synthesis and secretion (Rosen, Dalkin et al. 1991; Yasin, Dalkin et al. 1995). DNA sequence analysis of the GnRH-R promoter has identified 2 GSEs within 600bp of the start site, and the GSE identified between -250bp and -232bp was part of a tripartite, tissue specific enhancer conferring gonadotroph specific expression to the mouse GnRH-R (Clay, Nelson et al. 1995). This enhancer region includes an AP-1 site (White, Duval et al. 1999) and a GnRH-R activating sequence (GRAS) (Duval, Nelson et al. 1997). SF-1 has also been shown to interact with the GSE region to up-regulate GnRH-R mRNA expression levels (Ngan, Cheng et al. 1999). Activin and inhibin can also regulate the receptor number both at the transcriptional level through the GRAS region of the promoter (Fernandez-Vazquez, Kaiser et al. 1996; Duval, Ellsworth et al. 1999) and at the translational level (Braden and Conn 1992).

1.3.5 GnRH receptor uncoupling

The third mechanism that may be involved in differential regulation of the gonadotroph subunits is GnRH-R uncoupling, and activation of different second messenger pathways. The GnRH-R is a seven transmembrane

receptor coupled to the G proteins, G_q and G₁₁ (Hsieh and Martin 1992). There are a number of different signalling pathways that can be used by G protein coupled receptors. These include protein kinase C, mitogen activated protein kinase (MAPK), protein kinase A, and calcium influx. Many studies have been carried out to identify which pathways are involved in signalling differential transcription of the gonadotrophin subunits, either by different signalling pathways, (G protein uncoupling) or by unique pathways, which activate gonadotrophin subunit transcription individually. These are discussed in the following section and are summarised in Fig. 1.3.4.

1.3.5a Activation of Protein Kinase C and Protein Kinase A

Coupling of the GnRH-R to the G_q/11 protein initiates the phospholipase C (PLC) cascade. Hydrolysis of phosphatidylinositol 4,5-bisphosphate, forms inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG), which induces a rise in intracellular Ca²⁺, mobilizing both cellular and extracellular calcium pools (Naor, Leifer et al. 1980). This activates protein kinase C (PKC), causing a rapid secretion of LH. However inhibition of IP₃ does not inhibit secretion, suggesting that this pathway is not essential for secretion (Hawes, Marzen et al. 1992). PKC can also be uncoupled from GnRH stimulated LH secretion and has been suggested to have a role in modulating gonadotrope responsiveness to GnRH (Johnson, Mitchell et al. 1988). PKC has also been implicated in LH biosynthesis (Papavasiliou, Zmeili et al. 1986), and depletion of PKC in rat pituitary cell cultures inhibits the GnRH stimulated increase in LH β mRNA (Andrews, Maurer et al. 1988). GnRH-R can also couple to G_s, and G_i, activating protein kinase A (PKA) and cyclic adenosine

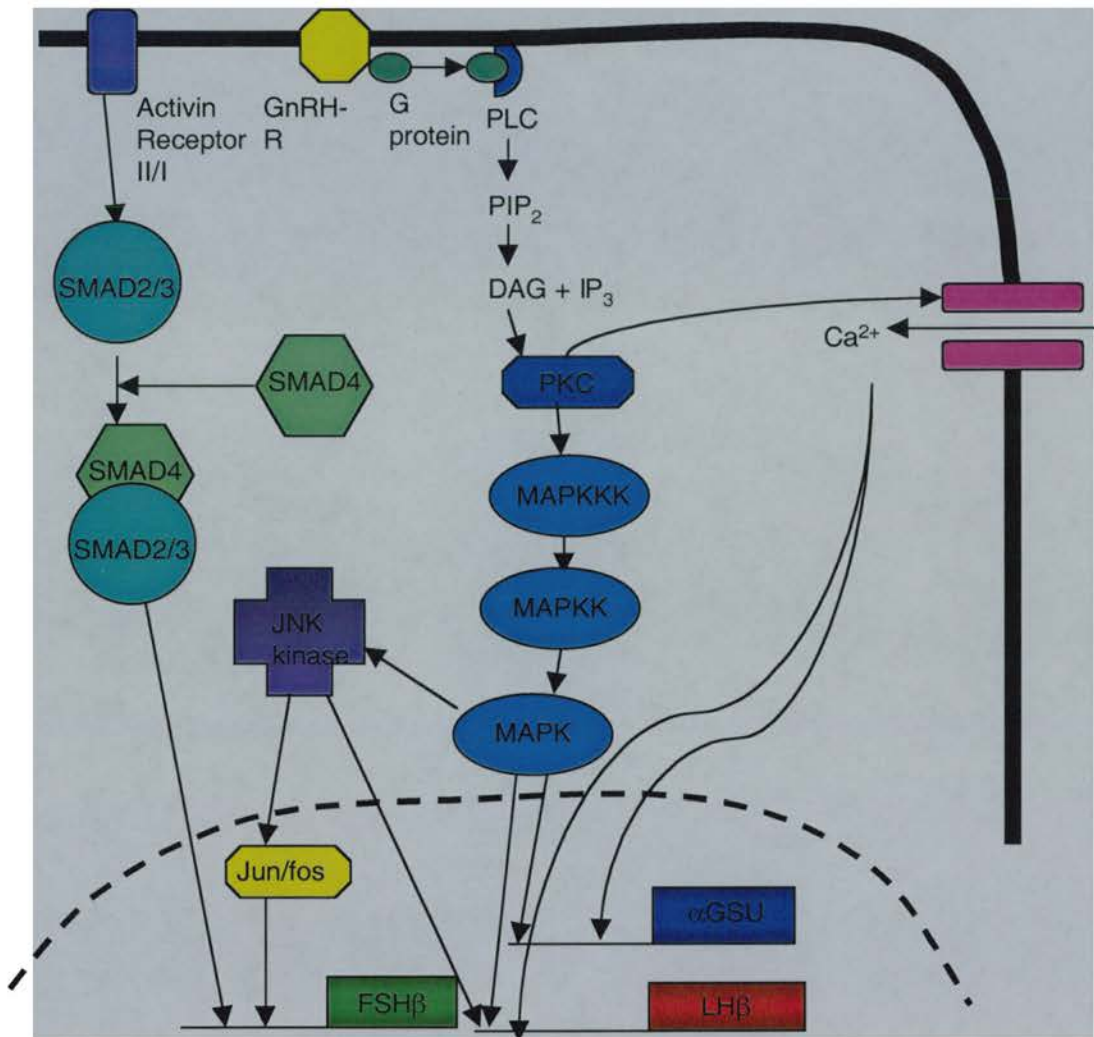


Fig.1.3.4: Schematic representation of the signalling pathways that may be involved in up-regulating transcription of the gonadotrophin subunits.

GnRH binds to and activates the GnRH receptor (GnRH-R) and releases the α subunit of the G protein. This binds and activates phospholipase C (PLC) which activates protein kinase C (PKC) via the diacylglycerol (DAG), inositol phosphate pathway (IP₃). PKC can then activate a number of other pathways including the mitogen activated protein kinase (MAPK) cascade, voltage gated Ca²⁺ ion channels and the jun N-Terminal kinase (JNK) cascade via jun/fos. The activin signalling mechanism through the SMAD proteins is also shown.

mono-phosphate (cAMP), although cAMP release has not been observed (Stanislaus, Pinter et al. 1998). The PKA pathway is possibly involved with increasing LH biosynthesis (Starzec, Moumni et al. 1989), regulating gonadotropin subunit mRNA (Ishizaka, Tsujii et al. 1993), or GnRH-R mRNA (Alarid and Mellon 1995).

The activation of PKC results in a number of different effects, one of which is to activate transcription. PKC itself is activated by DAG phosphorylation (Borner, Eppenberger et al. 1988; Pears and Parker 1991), and a recent review details a number of pathways that regulate PKC phosphorylation beyond that of DAG and calcium activation. This review suggests that a number of priming phosphorylations are required before activation takes place. These include phosphorylation of the activation loop site (Cazaubon, Bornancin et al. 1994; Hansra, Garcia-Paramio et al. 1999), the autophosphorylation site (Flint, Paladini et al. 1990), and the hydrophobic C-terminal site (Bornancin and Parker 1996; Bornancin and Parker 1997). At this point, ligand dissociation can occur, so that the kinase is held in a latent state, which can be recruited back to the membrane and reactivated by DAG (Parekh, Ziegler et al. 2000).

The existence of a primed PKC has implications for how a pulsatile GnRH signal is interpreted by the second messenger signal transduction systems. GnRH could differentially regulate a number of different pathways, including transcription and secretion, in particular transcription of α GSU and LH β subunit, and secretion of LH. As already described, α GSU transcription and LH secretion only require the presence of GnRH. At the LH surge, the pulse frequency of GnRH increases, mimicking a state of constant GnRH

administration, which has already been shown to favour transcription of α GSU. A change in GnRH-R coupling/signalling, possibly due to primed PKC, could possibly direct increased transcription of α GSU, but certainly stimulate secretion of LH.

The downstream targets of PKC are varied, and many are yet to be identified. Transcription of the gonadotrophin subunits, has been shown to be controlled by a PKC activated pathway (Cesnjaj, Catt et al. 1994). In this paper the early response gene expression profiles, in particular c-fos, c-jun and junB, were stimulated in response to GnRH. This response was rapid, the maximal effect occurring 30min after GnRH treatment, and was linked to the activation of the PKC pathway. Interestingly, sequence analysis of the ovine FSH β subunit has identified a number of potential activating protein-1 sites (AP-1). These AP-1 sites bind the jun/fos complex, up-regulating the transcription of FSH β (Strahl, Huang et al. 1997). By using a series of deletion constructs the authors were able to show that expression of the ovine FSH β gene was enhanced by c-jun, and c-fos proteins in transiently transfected mammalian cells. This was localised to 2 functionally linked elements which bound AP-1 proteins, and were also activated by 12-O-tetradecanoyl phorbol-13-acetate (TPA), a known activator of PKC.

Induction of α GSU gene expression is mediated through the PKC/mitogen activated kinase kinase (MEK) pathway (Weck, Fallest et al. 1998) and it has been suggested that LH β transcription is mediated through the PKC/MEK pathway (Saunders, Sabbagh et al. 1998). LH β mRNA expression is also increased with the addition of phorbol esters to a perifused rat pituitary

system (Ben-Menahem and Naor 1994), which lends credence to the argument that it is the PKC pathway rather than the calcium pathway that up-regulates LH β transcription. However the regulation of transcription may not be limited exclusively to one pathway or the other, especially when considering the transcriptional regulation of the gonadotrophin subunits.

1.3.5b Calcium Signalling Pathways

Although calcium-signalling pathways may have a possible role in the gonadotroph to signal transcription and secretion, the evidence is contradictory. It has been shown that calcium induces both transcription and secretion of α GSU (Holdstock, Aylwin et al. 1996), as well as inducing transcription of LH β (Ben-Menahem and Naor 1994; Weck, Fallest et al. 1998). A role for calcium in transcriptional regulation of c-fos has recently been shown (Hardingham, Chawla et al. 1997), but it is not clear if this could operate in gonadotrophs. Two separate signalling pathways could activate c-fos mediated transcription; one through the cAMP response element (CRE) by increasing the nuclear calcium levels and the other through the serum response element (SRE) by increasing levels of calcium in the cytoplasm and both signalling pathways can act independently. This has interesting possibilities for the control of FSH β mRNA transcription, which can be up-regulated by c-jun and c-fos.

1.3.5c Mitogen Activated Protein Kinase (MAPK) Activation

MAPK is the major downstream effector of GnRH. GnRH-R couples to either G $_i$ or G $_q$, activating PKA and PKC (Hawes, van Biesen et al. 1995), which activates MAPK in castrated testosterone replaced adult male rats

(Haisenleder, Cox et al. 1998), and in GH₃ cells stably transfected with GnRH-R (Han and Conn 1999).

Both α GSU and LH β gene expression levels are regulated by MAPK (Saunders, Sabbagh et al. 1998; Weck, Fallest et al. 1998). On the α GSU promoter, this regulation is likely to be mediated through an Ets binding site, since the Ets family of transcription factors are known to mediate transcriptional responses to the MAPK pathway (Maurer, Kim et al. 1999). There is also evidence for combinatorial cross-talk between PKC and Ca²⁺, which may be involved in fine-tuning the regulation of transcription by these pathways (Shacham, Harris et al. 2001).

1.3.5d Jun N-Terminal Kinase Pathway Activation

The Jun N-terminal kinase (JNK) pathway, has also been shown to be activated by GnRH through a PKC and tyrosine kinase dependent manner (Levi, Hanoch et al. 1998). The stimulation of the JNK cascade was shown to be greater, although slower than and independent of, the MAPK pathway and seemed to involve PKC, cSrc, CDC42/Rac1. L β T2 cells were identified as having a c-jun dependent JNK kinase (Yokoi, Ohmichi et al. 2000), but it is not known if the activation of the JNK cascade is sufficient to activate the LH β promoter.

1.3.6 Regulation of FSH β by gonadal peptide activation of TGF β signalling

The primary factors that regulate FSH synthesis and secretion, are the gonadal peptides activin, follistatin, and inhibin (Carroll, Corrigan et al. 1989). These were originally purified from porcine and bovine follicular fluids

(Ying 1988), and activin and inhibin are members of the transforming growth factor β (TGF β) family (Vale, Rivier et al. 1988). They bind and signal through serine/threonine kinase receptors (Mathews and Vale 1991; Massague 1998). Specifically, activin binds to the activin type II receptor, which recruits the type I receptor (Attisano, Wrana et al. 1996), and signals through the SMAD (SMADs are downstream components of serine/threonine kinase receptors) second messenger pathway to up-regulate transcription (Lebrun, Takabe et al. 1999). Follistatin binds to activin, preventing activin from interacting with the activin type II receptor, thus regulating its activity (Shimonaka, Inouye et al. 1991). Inhibin also binds the type II receptor, but cannot recruit the type I receptor, thus blocking activation of the second messenger system however, this does not completely inhibit activin activation (Martens, de Winter et al. 1997). Recently, evidence for an inhibin specific receptor has been reported. The betaglycan type III TGF β receptor can bind inhibin and act as an inhibin specific co-receptor with the activin type II receptor (Lewis, Gray et al. 2000). A membrane bound inhibin specific binding protein has also been isolated (p120), which may also interact with the activin type II receptor (Chong, Pangas et al. 2000), though its function is still unclear. However, mutations in the human p120 protein cause Pettigrew Syndrome, and human ovarian carcinomas (Choi, Cho et al. 1997; Lagerstrom-Fermer, Sundvall et al. 1997), the phenotype of which was recapitulated in a loss-of-inhibin transgenic mouse model (Matzuk, Finegold et al. 1992; Matzuk, Kumar et al. 1996).

1.4 GnRH regulated transcription of gonadotrophins.

GnRH activation of second messenger signalling is eventually transduced into transcriptional regulation of the gonadotrophin subunits. Progress in this field has been hampered due to the inaccessibility of gonadotroph cells in the anterior pituitary and due to the distribution of gonadotrophs in the pituitary. Gonadotrophs are evenly spread throughout the pituitary, so are difficult to isolate and dissection has not yielded primary gonadotroph cells or cell-lines, and human pituitary tumours yield a heterogeneous mix of pituitary cell-types. Therefore, GnRH regulated transcription has only recently been studied using *in vitro* transformed cell lines of the anterior pituitary and it was only with the advent of these cell-lines that the GnRH-R was cloned (Tsutsumi, Zhou et al. 1992). These cell lines were developed by the targeting of SV40 T antigen oncogene to gonadotrophs by the 5' flanking sequences of either the mouse α GSU or rat LH β gene promoter and injected into eggs. The resultant transgenic mice developed tumours of the anterior pituitary, from which transformed gonadotroph cells were derived. α T3-1 cells are able to synthesise and secrete α GSU, and express GnRH receptor, but do not synthesise either LH β or FSH β subunit (Windle, Weiner et al. 1990). L β T2 cells synthesise and secrete LH in response to GnRH (Turgeon, Kimura et al. 1996), and secrete FSH in response to activin A, thus proving that L β T2 cells are gonadotrophs (Graham, Nusser et al. 1999).

1.4.1 LH β transcription

A gonadotroph-specific transcription factor, which uniquely up-regulates LH β transcription has not been identified. Instead, the current hypothesis is

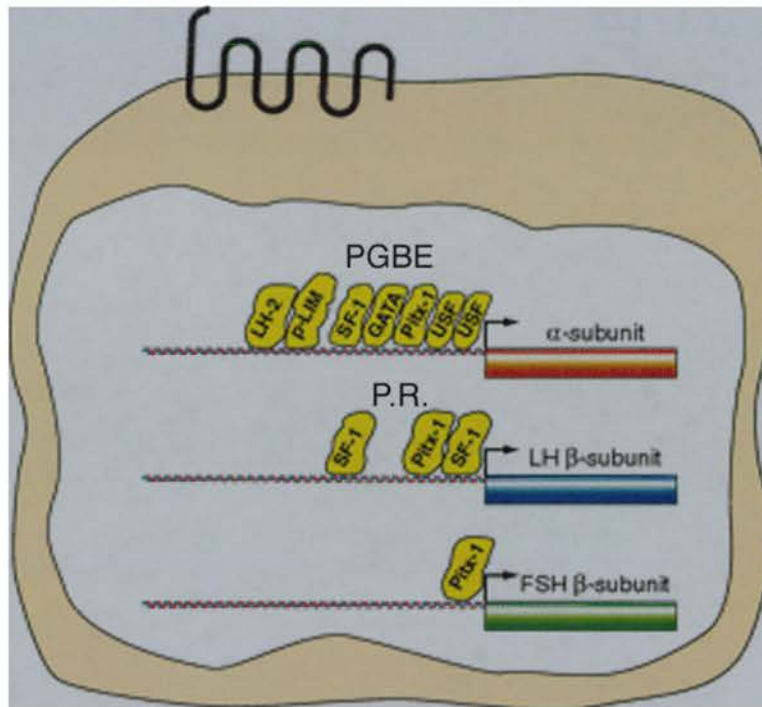


Fig. 1.4A: A schematic representation (not to scale) of the transcription factors that regulate basal transcription of the gonadotrophin subunit genes (adapted from Brown et al 1999). The PGBE region of the α GSU gene promoter and the proximal region (P.R.) of the LH β gene promoter are also shown.

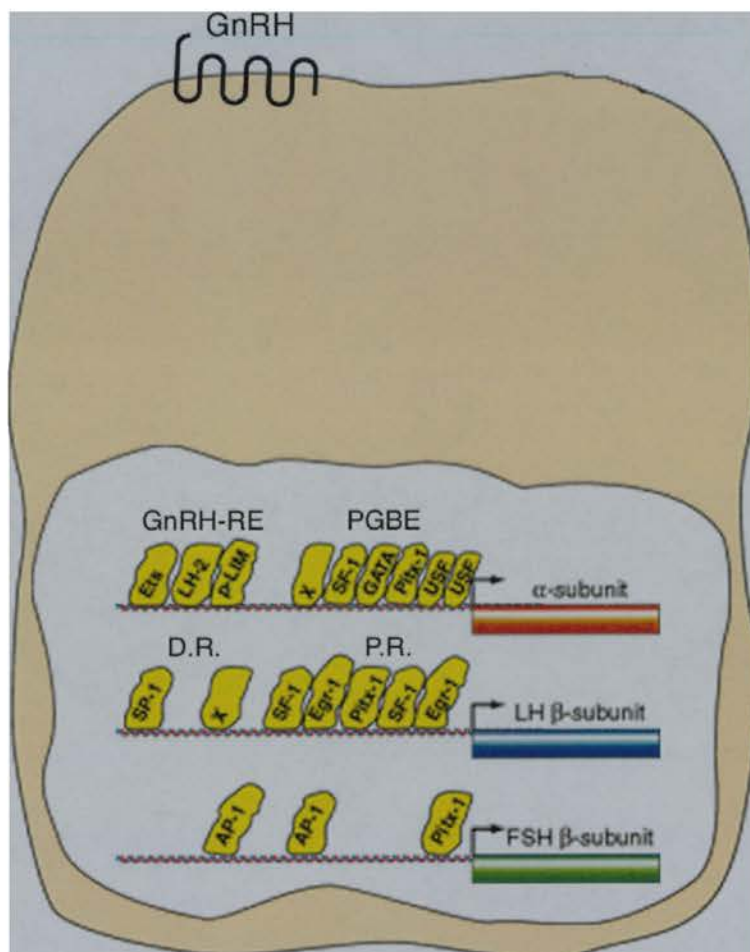


Fig. 1.4B: A schematic representation (not to scale) of the transcription factors involved in up-regulating transcription of the gonadotrophin subunits in response to GnRH (adapted from Brown et al 1999).

The GnRH-RE and the PGBE regions of the α GSU promoter, and the distal and proximal regions (D.R. and P.R.) of the LH β promoter are also included.

that transcription is up-regulated by a number of transcription factors, that are also expressed in other cell-types, these interact with each other and in this way provide combinatorial specificity (Brown and McNeilly 1999). Characterisation of the LH β promoter is most advanced in the rat. There are 2 regions involved in GnRH stimulated transcription, one at -490bp to -352bp (distal region), and the other maps between -207bp and -82 bp (proximal region). The distal region contains a GSE and a GnRH specific regulatory region (Kaiser 1998), which binds Sp1. In the proximal region, a number of other transcription factor binding sites have been identified, these are summarised in Fig 1.4 A and B. In addition a binding site for nuclear factor-Y (NF-Y) has been identified in the bovine LH β promoter (Keri, Bachmann et al. 2000) but the importance of this factor in regulation of transcription in other species is not known. The transcription factors regulated by GnRH, and shown to modulate LH β gene expression are now discussed in more detail below.

1.4.1a Sp1

Sp1 is a transcription factor that is ubiquitously expressed, and binds to a consensus DNA sequence of (G/T)GGGCGG(G/A)(G/A). Two Sp1 binding sites were identified, which acted synergistically (Kaiser, Sabbagh et al. 1998) in response to GnRH stimulation. However there was no change in the expression pattern or EMSA pattern of Sp1 either in the presence, or absence of GnRH. This implies that Sp1 is not regulated by GnRH and is constitutively active, in a similar fashion to SF-1. More recently, Sp1 has been identified as part of a tripartite GnRH response element (GnRH-RE), which also binds SF-1 and Egr-1 (see Fig. 1.4B). Interestingly, mutations in the Sp1

binding site do not affect basal or GnRH regulated LH β transcription, however mutating the Sp1, and Egr-1 binding sites does repress the GnRH regulated LH β promoter activity, and both are required to mediate the full GnRH response (Kaiser, Halvorson et al. 2000).

1.4.1b Egr-1 and synergistic factors

Egr-1 (NGFI-A, Krox-24) is a zinc finger transcription factor, initially identified as an immediate early serum response or nerve growth factor response gene product. Targeted disruption of the *Egr-1* gene in mice rendered females infertile due to lack of LH β gene expression (Lee, Sadovsky et al. 1996). The LH β promoter has potential binding sites for Egr-1 (DNA consensus sequence GCGTGGGCG), and is marginally up-regulated by Egr-1 in *in vitro* transfection assays, but co-transfection of SF-1 in this assay markedly increased this response (Lee, Sadovsky et al. 1996), indicating synergism between the factors. The synergism between Egr-1 and SF-1 is a major regulatory mechanism in the LH β promoter in a number of species (Lee, Sadovsky et al. 1996; Ito, Yu et al. 1997; Dorn, Ou et al. 1999; Wolfe and Call 1999). The original study by Lee et al has been expanded by a report of another *Egr-1* disrupted mouse with a wide-ranging phenotype (Topilko, Schneider-Maunoury et al. 1998). These transgenic animals have fewer somatotroph cells, but similar numbers of gonadotroph cells when compared to their wildtype littermates. So, Egr-1 regulates LH β transcription in gonadotrophs, and possibly cell survival in somatotrophs. There may also be redundancy between Egr-1 and another family member Egr-4, which can partially activate LH synthesis and explain how the males

were fertile in the earlier study, since Leydig cell steroidogenesis was maintained (Tourtellotte, Nagarajan et al. 2000).

Additionally, Egr-1 synergises with Pitx1 as well as SF-1. Unlike Pitx1 and SF-1, Egr-1 seems a likely mediator of GnRH induced signals for activation of the *LH β* gene, (Tremblay and Drouin 1999), and although transfection data in L β T2 cells suggests that mutating the Pitx1 site has no effect on the GnRH regulated activity of the *LH β* promoter (Quirk, Lozada et al. 2001), *in vivo* studies contradict this (Quirk, Lozada et al. 2001).

Egr-1 is responsive to GnRH. Addition of GnRH to cells leads to an increase in the mRNA levels of Egr-1 through activation of the PKC and MAPK pathways, therefore Egr-1 modulates *LH β* subunit gene transcription in a GnRH dependent manner (Dorn, Ou et al. 1999; Halvorson, Kaiser et al. 1999; Wolfe and Call 1999). It is likely that the action of Egr-1 is also modulated by GnRH, since it is known that Egr-1 protein has an internal inhibitory domain comprising a region of 34 amino acids that can bind a group of proteins known as Egr-1/NGF1-A binding proteins Nab 1 and Nab 2 (Russo, Sevetson et al. 1995; Svaren, Sevetson et al. 1996; Swirnoff, Apel et al. 1998). Nab1 mRNA was shown by Northern blot analysis, to be regulated by GnRH, and may therefore be a factor involved in regulating Egr-1 action (Wolfe and Call 1999).

1.7.1 FSH β transcription

1.4.2a Regulation of FSH β by gonadal peptides

The gonadal peptides activin and inhibin are also locally synthesised in the anterior pituitary in response to GnRH (Kirk, Dalkin et al. 1994; Dalkin, Haisenleder et al. 1998) and are involved in regulating expression of the *FSH β* gene via the SMAD proteins (Dalkin, Haisenleder et al. 1999). Activin up-regulates FSH β by stabilising the mRNA (Carroll, Corrigan et al. 1991) and by increasing transcription (Weiss, Guendner et al. 1995). Inhibin and follistatin both down-regulate FSH β and α GSU mRNA levels (Attardi and Winters 1993; Pernasetti, Vasilyev et al. 2001), and can inhibit FSH secretion (McNeilly 1988). Inhibin, in particular, can decrease the transcription rate of the *FSH β* subunit gene (Clarke, Rao et al. 1993). Activin and inhibin are known to regulate *FSH β* transcription *in vivo* (Carroll, Corrigan et al. 1989), and it has recently been shown that addition of activin to L β T2 cells, will activate transcription of *FSH β* *in vitro* (Graham, Nusser et al. 1999). Activin binds to, and activates the type II activin receptor, which recruits the type I receptor (Mathews and Vale 1991). This phosphorylates and activates SMAD 2/3, which interacts with SMAD4 and translocates to the nucleus. The SMAD complex can then bind DNA and up-regulate transcription (Lebrun, Takabe et al. 1999).

1.4.2b Regulation of FSH β through Activating Protein-1 sites

Sequence analysis of the ovine FSH β promoter has identified a number of putative activating protein-1 (AP-1) sites with near or identical homology to the consensus AP-1 site (TGA^G/_CTCA). As described in section 1.3.4a, these AP-1 sites are functionally linked to up-regulate the FSH β gene promoter, mediated by c-jun and c-fos (Strahl, Huang et al. 1998). There is additional evidence to suggest that Jun and Fos proteins may be involved in FSH β transcription since activin was able to up-regulate Jun and Fos mRNA levels *in vitro* (Hashimoto, Gaddy-Kurten et al. 1993). The importance of these AP-1 sites in up-regulating FSH β promoter was investigated in transgenic mice carrying either the wild type ovine FSH β promoter, or an ovine FSH β promoter with mutated AP-1 sites (Huang, Sebastian et al. 2001). The results showed that the GnRH response of the FSH β promoter acted through the AP-1 sites, however, activin was the major regulator of FSH β promoter activity, and it did not act through the AP-1 sites (Huang, Sebastian et al. 2001). A DNA binding site for the activin activated SMAD2/3/4 complex has yet to be identified, however a recent paper has identified that several anterior nuclear proteins can bind to an -800bp upstream region of the TATA box, it is perhaps in this region that an activin effector site will be identified (Kato, Tomizawa et al. 1999).

1.4.2c Regulation of FSH β synthesis by Bone Morphogenic Proteins

There may also be a role for bone morphogenic proteins (BMP) in the synthesis of FSH. BMP-6 and BMP-7 mRNAs have been detected in mouse

pituitaries and L β T2 cells (Huang, Wu et al. 2001). BMPs belong to the same superfamily of transforming growth factor- β (TGF- β) proteins as activin, and were originally identified by their ability to induce bone and cartilage formation (Wozney 1989). When transfected with the ovine FSH β promoter, BMP-6 and BMP-7 both up-regulate FSH β transcription (Huang, Wu et al. 2001) but their site of action has yet to be identified. The known transcription factor binding sites have been summarised in Fig 1.4 A and B.

1.4.4 α GSU transcription

1.4.4a Pituitary Glycoprotein Hormone Basal Element

α GSU mRNA up-regulation by GnRH is mediated by the MAPK pathway (Roberson 1995) and mobilisation of extracellular Ca²⁺ (Holdstock, Aylwin et al. 1996). Sequence analysis of the α GSU promoter identified two regions that are involved in GnRH regulated α GSU expression (Schoderbek, Roberson et al. 1993), these have been designated as the pituitary glycoprotein hormone basal element (PGBE) and the GnRH responsive element (GnRH-RE). In addition to a SF-1 and Pitx1 binding site, the PGBE region contains two E-boxes α EB1 and α EB2, at -51bp to -45bp and -21bp to -16bp respectively, which can bind helix-loop-helix proteins. α EB2 can bind upstream stimulatory factor (USF), which has been previously shown to up-regulate SF-1 transcription (Harris and Mellon 1998) and is required for maximal up-regulation of the α GSU promoter (Jackson, Gutierrez-Hartmann et al. 1995). The transcription factors that bind to the PGBE are summarised in Fig. 1.4A.

1.4.4b GnRH Responsive Element

Further analysis of the GnRH-RE identified a core Ets binding site (GGAA), which, when mutated, inhibits GnRH responsiveness of the α GSU promoter. Experiments identified that Ets-2 could bind to this region and initiate a GnRH response (Roberson 1995). Lhx2 has also been implicated in mediating GnRH regulated transcription since it interacts with the Ets transcription factor, and synergises up-regulation of α GSU expression (Glenn and Maurer 1999). Ca^{2+} mobilisation also induces an as yet unidentified protein, which binds to a -420bp and -224bp region of the α GSU promoter and also up-regulates the GnRH response (Holdstock, Aylwin et al. 1996). The up-regulation of α GSU expression by different signalling mechanisms, therefore ensures adequate amounts of α GSU over LH or FSH β -subunits. The transcription factors that bind to both the PGBE and the GnRH-RE are summarised in Fig. 1.4B.

1.4.5 Steroid hormone regulation of transcription

In females, LH and FSH levels are also regulated by oestradiol. Animals that were gonadectomised to remove the negative feedback effects of steroid hormones, showed increased mRNA levels of LH β , FSH β , and α GSU (Gharib, Bowers et al. 1986). Oestradiol was shown to inhibit transcription of all three gonadotrophin subunits, specifically LH β (Shupnik, Gharib et al. 1988). Further experiments on normal, OVX and ovariectomised-oestradiol replaced rats produced conflicting results. In the pituitaries of OVX rats either left untreated or treated with oestradiol, LH β mRNA levels were

increased in response to oestradiol. In the pituitaries of normal rats, oestradiol treatment down-regulated LH β mRNA expression levels (Shupnik, Gharib et al. 1989). However, a positive steroid response was mediated by a region on the LH β promoter which was able to bind the oestrogen receptor (Shupnik, Weinmann et al. 1989). This region may be responsible for the positive feedback of oestradiol on LH synthesis and secretion during the preovulatory LH surge and may explain the divergent effects seen in the pituitaries seen in the OVX, and non-OVX rats. Oestradiol treatment of GnRH treated L β T2 cells enhanced the mRNA levels of LH β and this was attributed to an increase in GnRH-R number (Turgeon, Kimura et al. 1996). Oestradiol treatment of the hypogonadal mouse did not show any significant increase in LH β or FSH β mRNA levels but did increase α GSU mRNA levels, however the authors suggest that the increase in mRNA level was not due to an increase in transcription rate (Stanley, Lyons et al. 1988).

In vivo studies in OVX mice suggested that the increase in FSH β mRNA and FSH serum levels was not due to an increase in FSH β transcription rate and that inhibin may have a non-transcriptional role in regulating FSH β mRNA levels (Dalkin, Knight et al. 1993). FSH β mRNA levels are also decreased in response to oestradiol treatment, and a study has shown that oestradiol and progesterone decrease steady state mRNA levels in ovine pituitary cultures (Phillips, Lin et al. 1988). *In vitro* studies, using primary cultures of sheep pituitaries has identified a region of the ovine FSH β promoter between -105bp and -72bp, which can repress transcription when stimulated with oestradiol. This repression is mediated by oestrogen receptor/protein interactions with basal transcription factors (Miller and Miller 1996).

1.5 LH and FSH secretion

1.5.1 Regulation of secretion

The gonadotrophs differentially secrete LH and FSH, in response to GnRH through constitutive and regulated secretory pathways (Muyan, Ryzmkiewicz et al. 1994). LH and FSH are normally packaged into separate secretory granules and the LH containing granules are secreted mainly through the regulated pathway, while the FSH containing granules are secreted through the constitutive pathway (Farnworth 1995), however some granules contain both LH and FSH. The granins are acidic proteins that are found in endocrine secretory granules (Winkler and Fischer-Colbrie 1992) and have been implicated as sorting proteins since they are present in the secretory granules of gonadotrophs (Deftos 1991; Watanabe, Uchiyama et al. 1991). These granins are known as chromogranin A (CgA), chromogranin B (CgB) and secretogranin II (SgII), and may have a role in the regulated secretion of gonadotrophins.

GnRH, after primary application, can alter the responsiveness of gonadotrophs so that further treatment with GnRH induces greater LH secretion. This is known as the priming effect (Aiyer, Chiappa et al. 1974; Fink, Chiappa et al. 1976; Pickering and Fink 1979) and acts through the PKC pathway that initiates the release of arachadonic acid via the up-regulation of phospholipase A₂ (Thomson, Johnson et al. 1994). *In vitro* treatment of mouse pituitaries with GnRH induced this priming effect, secretory granules were translocated to subplasmalemmal regions around the cell circumference, which would allow greater LH release when the cells were

further exposed to GnRH, (Lewis, Morris et al. 1986). This is thought to be mediated by changes of the microfilaments of the cytoskeleton (Lewis, Morris et al. 1985). Transmission electron microscopy (EM) of sheep pituitaries throughout the oestrus cycle revealed that only granules between 130nm and 150nm in diameter migrated through the cytoplasm to the plasma membrane close to blood vessels (these cells are described as being polarised). The number of polarised cells increased throughout the follicular phase so that during the LH surge, 90% of gonadotrophs were polarised. After the LH surge, the gonadotrophs were almost devoid of LH granules, those that remained were 300nm in diameter, demonstrating the preferential exocytosis of the smaller granules (Currie and McNeilly 1995). This suggests that the preovulatory LH surge is due to increased numbers of polarised gonadotroph cells, rather than an increase in LH synthesis. This priming effect is concomitant with an increase in GnRH-R numbers and mRNA levels (Brooks, Crow et al. 1992), and is enhanced by oestradiol (Thomas and Clarke 1997). The larger granules show immunoreactivity for LH, FSH and CgA, however the smaller granules only contain LH and SgII (Watanabe, Uchiyama et al. 1991). Immunocytochemistry of gonadotrophs after oestradiol treatment also identified that the polarised LH granules only contained LH and SgII (Thomas, Takahashi et al. 1998). This is evidence of a role for SgII in exocytosis of small LH granules at the LH surge.

Studies of granules within chromaffin cells has identified that out of 20000 granules, only 1000 are within 10nm of the plasma membrane (Steyer, Horstmann et al. 1997). Actin may act as a barrier between the granules and the plasma membrane. Investigation in cultured lactotrophs of female rats identified that the F-actin network dissociated with the addition of

secretagogues such as forskolin and the secretory granules moved to the plasma membrane (Carbajal and Vitale 1997) possibly via myosin motor activation (Mooseker and Cheney 1995). Inhibition of this dissociation inhibited secretion of prolactin (Carbajal and Vitale 1997). Recently Ca^{2+} has been shown to disassemble the F-actin network in GH3 cells, and substantiates the hypothesis that F-actin acts as a barrier to exocytosis, and postulates that Ca^{2+} directly controls cytoskeletal changes (Yoneda, Nishizaki et al. 2000). Thus the actin barrier may have a role in the storage and regulated secretion of LH granules by preventing premature secretion of LH (Fig. 1.5.1).

FSH secretion is independent of GnRH (Farnworth 1995), but is regulated by activin, which increases FSH secretion. Inhibin and follistatin, which also inhibit FSH β transcription, regulate activin activity and hence secretion (Schwall, Nikolics et al. 1988; Carroll, Corrigan et al. 1989). Additionally, FSH synthesis appears to be closely linked to secretion after GnRH treatment (Brooks, Crow et al. 1992). Polarisation of FSH granules to the plasma membrane has not been shown, instead, the granules remain dispersed throughout the cytosol (Thomas and Clarke 1997).

1.5.2 Exocytosis

Much of what is currently known about neuroendocrine exocytosis has been shown in chromaffin cells. A number of key proteins, which have a role in exocytosis have been identified. Rab3 is a member of a family of membrane associated guanine nucleotide binding proteins with GTPase activity, that are components of the regulated exocytosis pathway



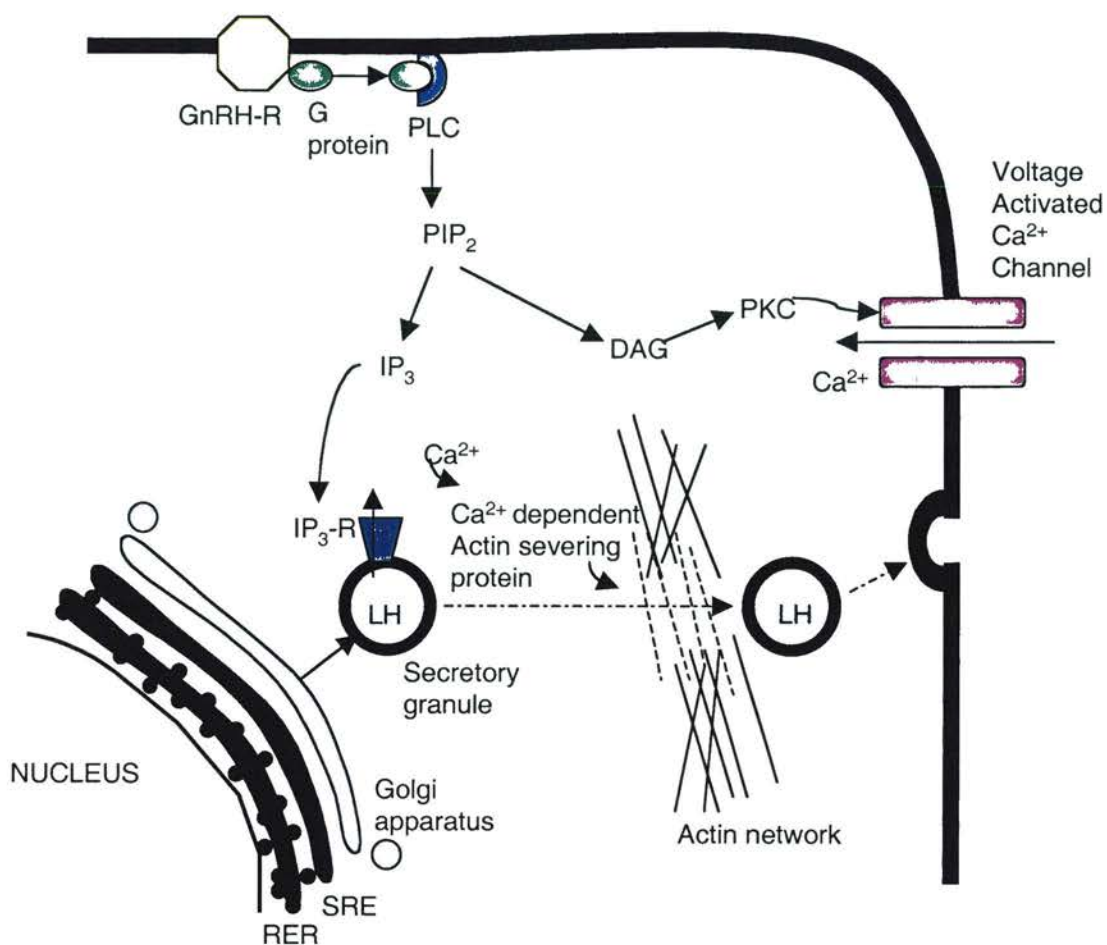


Fig. 1.5.1: Schematic representation of GnRH induced secretion including the signalling pathways involved. GnRH activates the GnRH receptor (GnRH-R), which activates the α subunit of the G protein. This activates phospholipase C (PLC), which cleaves phosphoinositide 4,5 biphosphate (PIP₂), creating DAG and IP₃. Both DAG and IP₃ increase intracellular Ca²⁺ levels. This activates the Ca²⁺ dependent actin severing protein, which enables the granule to migrate to the plasma membrane. The secretory granule then fuses with the membrane and releases LH.

(Novick and Brennwald 1993). GnRH stimulated exocytosis is dependent on a Rab3 isoform, Rab3b (Tasaka, Masumoto et al. 1998), possibly by interacting with membrane associated proteins (Lledo, Johannes et al. 1993; Lledo, Johannes et al. 1994). These proteins include soluble *n*-ethylamide-sensitive factor attachment protein receptors (SNARE), which are found on vesicle membranes (vSNARE) and on target membranes (tSNARE). The two SNAREs interact, establishing contact between the vesicle membrane and the target membrane. These fuse and the contents of the vesicle are released, after the binding of the fusion molecules *n*-ethylamide-sensitive factor (NSF) and soluble *n*-ethylamide sensitive factor attachment proteins (SNAPs) to the SNARE complex (Burgoyne and Morgan 1993). This is summarised in Fig. 1.5.2. Exocytosis is a calcium dependent response, which in gonadotrophs, is released from intracellular Ca^{2+} stores, not from voltage activated extracellular Ca^{2+} influx (Tse, Tse et al. 1993; Tse and Tse 1999).

Currently, there has only been one report about exocytosis in the gonadotroph cell-line L β T2, which suggested that oestradiol increased the exocytotic response by sensitising the fusion machinery (Thomas, Mellon et al. 1996).

1.6 Summary

This chapter has described the physiological role of the gonadotrophins and has highlighted the importance of regulated LH and FSH synthesis and secretion. GnRH, a decapeptide released in pulses from the hypothalamus, controls this regulation and has a number of targets within the gonadotroph. It binds to and activates GnRH-R, a G protein coupled receptor, which

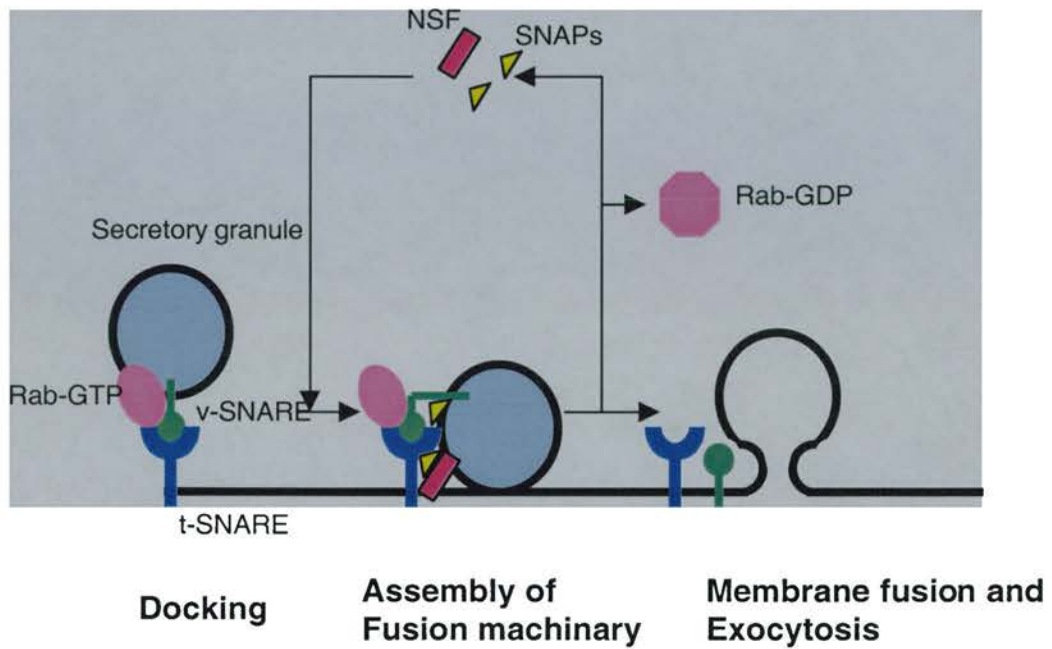


Fig. 1.5.2: A diagram showing the predicted mechanism of exocytosis in neuroendocrine cells.

The vesicle SNARE (v-SNARE) docks with the target SNARE (t-SNARE), which allows n-ethylamide sensitive factor (NSF) and soluble n-ethylamide sensitive factor attachment proteins (SNAPs) to bind. These catalyse the fusion of the vesicle membrane and the plasma membrane, converting Rab-GTP to Rab-GDP.

This diagram is adapted from Molecular Biology of the Cell, 3rd edition, Bruce Alberts.

activates PKC, this then activates a number of other second messenger pathways, some of which have been elucidated. They include the MAPK cascade, JNK kinase cascade, and Ca^{2+} signalling. The LH β , FSH β and α GSU subunits are differentially regulated by different pulse frequencies and this may be mediated by one of the pathways mentioned. The exact pathways that regulate transcription of each of the gonadotrophin subunits are yet to be defined. α GSU mRNA levels are increased by all frequencies of GnRH pulses, this ensures that α GSU protein is synthesised in excess of both FSH β and LH β proteins. LH β mRNA is more tightly regulated, since up-regulation only occurs with fast pulses of GnRH and FSH β mRNA is up-regulated by slow pulses of GnRH. In fact, regulation of FSH β mRNA levels appears to be mediated by the gonadal peptides activin, inhibin and follistatin. Inhibin and follistatin, down-regulate FSH β mRNA levels and activin up-regulates mRNA levels. These act through type I and type II activin receptors via SMAD proteins, although there is evidence to suggest that inhibin can also act through a betaglycan receptor. Differential regulation of transcription occurs by binding of different transcription factors. Although a gonadotroph specific transcription factor has yet to be identified, current evidence suggests that a number of common transcription factors bind to the promoters of the gonadotroph subunits in different combinations. GnRH regulated transcription appears to be up-regulated by a different sub-set of transcription factors, which again are not specific to gonadotrophs. GnRH also controls secretion of FSH and LH. FSH is secreted through the constitutive secretory pathway, and secretion is closely linked to transcription. LH secretion however, seems to be dissociated from LH β transcription and follows a Ca^{2+} dependent regulated secretory pathway.

A large number of studies have contributed in understanding the physiological roles of LH and FSH and how they are differentially regulated. The evidence points to a complex mechanism of intracellular signalling pathways and proteins that facilitate regulation of the gonadotrophins.

1.7 Aims of this Thesis

The main aim of these studies is to identify factors that are GnRH regulated, with particular focus on factors that may be involved in regulating LH and FSH synthesis and secretion. L β T2 cells are a fully differentiated gonadotroph cell-line that can synthesise and secrete LH and FSH. Therefore L β T2 cells have all the mechanisms required for regulating gonadotrophin hormone biosynthesis and secretion. Since neither these mechanisms nor the factors that are GnRH regulated are fully elucidated, differential display RT-PCR (Liang and Pardee 1992) will be used to identify and isolate differentially expressed mRNA transcripts from L β T2 cells (Turgeon, Kimura et al. 1996) that have been left untreated, or have been treated with GnRH.

The differentially regulated transcripts will then be cloned, sequenced, identified and characterised. Using DD-RT-PCR, it is hoped that a profile of GnRH regulated factors can be produced, that will add to the current information available about the downstream effects of GnRH-R activation. Full length clones of potential regulatory factors will then be used in transfection assays to identify if they are involved in regulating gonadotrophin subunit gene expression.

1.8 Layout of this Thesis

Chapter 2 has two parts, chapter 2a will detail the general materials and methodologies that were used throughout this project. Chapter 2b describes the materials and methodologies employed in the differential display RT-PCR technique, and the modifications made to this technique during the project. It will be presented as a mini-results chapter. Chapter 3 and 4 are the main results chapters. Chapter 3 describes the factors that were isolated by DD-RT-PCR, and their initial characterisation. Chapter 4 describes the results of further analysis of one of these factors, and its role in regulating gonadotrophin gene expression. Chapter 5 is a general discussion of the factors that have been identified, their possible roles in the gonadotroph, and how these roles can be further elucidated in future studies.

Chapter 2a:

General Materials and Methods

2.1 Introduction

This chapter describes the general molecular biology methods used, with a separate section for Differential Display RT-PCR (Chapter 2b), and the modifications made to this procedure. A complete list of suppliers can be found in Appendix I. Control of Substances Hazardous to Health (COSHH) safety guidelines for the handling of hazardous chemicals and bio-hazardous materials were followed.

2.2 General Materials

General Chemicals were purchased from Merck-BDH (UK), Roche (UK) or Sigma-Aldrich (UK), and were Analar grade. All water was double distilled and deionised before autoclaving.

The tables below contain details of specialised materials, grouped into general areas of methodology.

Table 2.2a: Bacterial Strains and Materials

Bacterial strains and Materials	Genotype	Supplier
JM109 cells	<i>EndA1 recA1 gyrA96 thi hsdR17 (r_K⁻, m_K⁺) relA1 supE44 Δ(lac-proAB) [F' proAB lacI^q ZΔM15]</i>	Promega (UK)

TOP10 One Shot™ Chemically Competent Cells	<i>F- mcrA Δ(mrr-hsdRMS- mcrBC) φ80lacZΔM15 ΔlacX74deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG</i>	Invitrogen (UK)
NovaBlue Singles™	<i>endA1 hsdR17 (r_{K12}⁻ m_{K12}⁺) supE44 thi-1 recA1 gyrA96 relA1 lac [F' proA⁺B⁺ lacI^qZΔM15::Tn10 (Tc^R)]</i>	Novagen (UK)
Luria Bertani (LB) Broth and Agar		Anachem (UK)
Kanamycin		Sigma-Aldrich (UK)
Ampicillin		Sigma Aldrich (UK)
S.O.C. medium		Gibco-PRL (UK)
5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-Gal)		Roche (UK)
Isopropyl β-D-thiogalactopyranoside (IPTG)		Roche (UK)
Miracloth		Calbiochem (UK)
Ultracentrifuge tubes		Kendro (UK)

Table 2.2b: Sources of clones and Vectors

Genetic Material	Size of Plasmid	Source
pcDNA 3	5.4kb	Invitrogen (UK)
pA3LUC	6.36kb	W.M. Wood (Denver, USA)
pBluescript (SK+/-)	2.96kb	Stratagene (UK)

pGEM®-4	2.87kb	Promega (UK)
pTAL-Luc	5kb	Clontech (UK)
pnlacF	6.3kb	Jacques Peschon (Seattle,US)
pp1786/pnlacF	8kb	P. Brown (MRC)
dLH2/pnlacF	7kb	P. Brown
dLH5/pnlacF	6.9kb	P. Brown
α 480/pA3LUC	6.84kb	P. Brown
α 120/pA3LUC	6.48kb	P. Brown
α 109/pA3LUC	6.47kb	P. Brown
FAA/pCEP 4	14.9kb	H. van de Vrugt (Amsterdam)
pT7-Blue	2.89kb	Novagen (UK)
pst-Blue-1	3.85kb	Novagen (UK)

Table 2.2c: Molecular Biology

Kit/ Reagent/ Enzyme	Supplier
Ready to Go™ T4 DNA Ligase	Amersham
Mobio Mini prep	Cambio (UK)
Jet Star 5 mini prep	AMS Biotechnology (UK)
Qiagen Maxiprep	Qiagen (UK)
Miniprep	Sigma (UK)
Big Dye Terminator II	Applied Biosystems (UK)

Direct Protect Lysate Ribonuclease Protection Assay Kit	Ambion (UK)
T7 Sequencing Kit	Amersham-Pharmacia (UK)
AGS Gold Taq Polymerase	Hybaid (UK)
Extensor Hi-Fidelity PCR Master Mix	ABGene (UK)
Perfectly Blunt Cloning Kit	Novagen (UK)
First Strand cDNA Synthesis Kit	Amersham Pharmacia (UK)
Hybond-N	Amersham Pharmacia (UK)
Restriction Enzymes	Roche (UK)
Shrimp Alkaline Phosphatase	Roche (UK)
GTG Agarose	FMC (UK)
Bio-Rad Protein Assay	Bio-Rad (UK)
RNAzol™ B	AMS Biotechnology (UK)
HYBAID RECOVERY™ DNA Purification Kit II protocol	Hybaid (UK)
T4 Polynucleotide Kinase	Roche (UK)
Oligonucleotides	Sigma-Genosys (UK)
Rediprime™ II random prime labelling kit	Amersham-Pharmacia (UK)
³⁵ S α dATP 1000Ci/mmol	Amersham-Pharmacia (UK)
³² P α dCTP 3000Ci/mmol	Amersham-Pharmacia (UK)
³² P γ dUTP 800Ci/mmol	NEN (UK)
¹⁴ C dithreo-Chloramphenicol 50-62mCi/mmol	Amersham-Pharmacia (UK)

Table 2.2d: Mammalian Cell lines and materials

LβT2 mouse gonadotroph cells	P. Mellon (San Diego, USA)
αT3-1 cells	P. Mellon (San Diego, USA)
Fugene™	Roche (UK)
GenePorter™	Gene Therapy Systems (UK)
Dulbecco's Modified Eagle's Media (DMEM)	Sigma (UK)
Phosphate Buffered Saline (PBS)	Sigma (UK)
Dimethyl sulfoxide (DMSO)	Sigma (UK)
Trypsin	Sigma (UK)
Penicillin/Streptomycin	Sigma (UK)
Fetal Calf Serum (FCS)	Sigma (UK)
Matrigel™	Becton Dickinson Labware (UK)
Culture flasks 25cm ² and 75cm ²	Costar (UK)
12 well plates	Costar (UK)/Nunc (UK)
Gonadotrophin Releasing Hormone (GnRH)	Peninsula (UK)
Cell scrapers (rubber policeman)	Nalge Nunc (UK)

2.3 General Molecular Biology Methods

2.3.1 Restriction Endonuclease Digestion

Restriction endonuclease digests were set up according to manufacturer's instructions, using supplied buffers, ensuring that the volume of enzyme did not exceed 10% (v/v) of the total reaction volume to minimise star activity, particularly when multiple digests were performed. In general, the digests were performed with 2-3 units of restriction enzyme per μg of DNA, and were digested for at least 2 hours.

2.3.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to resolve and analyse different sizes of DNA fragments after restriction endonuclease digestion or polymerase chain reaction (PCR). Tris-borate electrophoresis buffer (1xTBE pH 8.3; 90mM Tris, 90mM boric acid, 20mM EDTA pH8) was used for separation of small DNA fragments (typically <1000bp), and Tris-acetate electrophoresis buffer (1xTAE pH 7.7: 40mM Tris.Acetate, 2mM EDTA) was used for separation of large DNA fragments (>1000bp). The percentage agarose varied from 0.8% to 2% (w/v) and the gels were electrophoresed at 8-10V/cm. Ethidium bromide was added at a final concentration of 0.5 $\mu\text{g}/\mu\text{l}$. The agarose gel was trans-illuminated with low wavelength (302nm) UV light (UV transilluminator 2000, BioRad), to visualise the DNA.

2.3.3 Native Polyacrylamide gel electrophoresis (PAGE)

This technique was used to distinguish the cloning of a 45bp oligonucleotide into a plasmid. Colonies were picked and mini preps grown from agar plates. The DNA was extracted, and 20 μl was digested with *BanI* (Roche). A

12% acrylamide (19:1 acrylamide:bis-acrylamide) gel was prepared (Sambrook et al 1989) and the complete digest was loaded and electrophoresed at 12V/cm for 2 hours. The gel was stained with ethidium bromide (10µg/ml) in 1xTBE buffer, for 10 minutes, and destained in water for 5 minutes. The bands were visualised under UV light, and the positive clones identified.

2.3.4 Purification of restriction digested DNA

Agarose gels were used to fractionate DNA prior to purification. At least 30µg of plasmid DNA was digested overnight in a 100µl reaction volume with the specific enzymes to generate the required fragment. A small fraction of the reaction was electrophoresed on an 8cm agarose gel to check if the plasmid had cut to completion. The reaction was frozen to inactivate the enzyme, and loaded onto a 20cm gel with either 6x Orange G dye (15% Ficoll, 0.05% SDS, 20mM EDTA pH8, 0.15% Orange G), or 10x DNA loading dye (50% glycerol, 0.42% bromophenol blue, 0.4% xylene cyanol FF). The gel was electrophoresed overnight at 1.5V/cm. The fragment was visualised by ethidium bromide staining as described earlier, and the band of interest was cut from the gel with a sterile scalpel blade, and purified from the agarose following the Hybaid recovery DNA purification kit II protocol (Hybaid).

2.3.5 Purification of annealed oligonucleotides on a native

PAGE gel

The reaction was set up so that the final annealed oligonucleotide concentration was 1µg/µl (5µl of each oligonucleotide (4µg/µl), 2µl annealing buffer (140mM Tris pH 7.6, 14mM MgCl₂, 10mM DTT), 8µl water to give a

final reaction volume of 20 μ l). The oligonucleotides were heated at 100°C for 10 minutes and allowed to cool slowly back to room temperature. The annealed oligonucleotide was loaded onto a 12% acrylamide (19:1 acrylamide:bisacrylamide) gel, run at 160V for 2 hours and stained with 10 μ g/ μ l ethidium bromide. The oligonucleotide was excised from the gel and placed into 300 μ l of DNA elution buffer (20mM Tris.HCl pH 8, 400mM NaCl, 2mM EDTA pH8, 0.5% SDS) and incubated overnight at 37°C. After adding 700 μ l of 100% ethanol and 1 μ l of 10mg/ml dextran sulphate, the eluted DNA was precipitated at -70°C for 30 minutes. The DNA was pelleted by centrifugation at 13000 rpm for 20 minutes, and reconstituted in 100 μ l STE (0.3mM NaCl, 10mM Tris pH7.4, 1mM EDTA pH 8). The DNA was quantified spectrophotometrically (see section 2.3.8).

2.3.6 Deproteination of DNA by Phenol Chloroform Extraction

Proteins were removed from DNA by phenol chloroform extraction. A half volume of Tris-HCl (pH7.4) saturated phenol was added to the DNA sample, and mixed vigorously. Then a half volume of chloroform was added, and mixed. The tube was centrifuged at 13000rpm in a bench top centrifuge for 10 minutes to separate the layers, and the top layer was transferred to a fresh tube. One volume of chloroform was added, to remove the last traces of phenol, and the tube was vortexed and centrifuged again. The top layer was transferred to another fresh tube ready for ethanol precipitation.

2.3.7 Ethanol Precipitation of Nucleic Acids

Ethanol precipitation was used to purify and concentrate the DNA or RNA. Typically, 1/10 of the sample volume of 3M sodium acetate pH 5, and 2.5 to 3 times the sample volume of 100% ethanol was added to the DNA. This was mixed and stored at either -20°C for >1 hour or -70°C for >20 minutes. If the DNA sample was dilute, a co-precipitant of 1µl of 10mg/ml dextran sulphate, or See-DNA (Amersham) was added. Yeast tRNA (5mg/ml, Ambion) was used to co-precipitate RNA. The sample was then centrifuged at 13000rpm for 10-15 minutes to pellet the nucleic acid, then washed with 70% ethanol (optional), re-spun, and allowed to air dry. DNA was re-suspended in an appropriate volume of TE (10mM Tris pH7.4, 1mM EDTA pH 8), and RNA was re-suspended in DEPC treated water.

1.8.1 Spectrophotometrical Analysis of Nucleic Acids

Spectrophotometric analysis was performed using GeneQuant™ (Amersham-Pharmacia). At 260nm, an OD reading of 1 is equivalent to 50µg/ml of double-stranded DNA or 40µg/ml of RNA or single-stranded DNA. An OD reading at 280nm indicates the presence of other high molecular weight molecules such as proteins. A high quality DNA or RNA preparation should possess a 260nm:280nm ratio of 1.8 or 2 respectively.

2.3.9 Cloning

2.3.9a Dephosphorylation of linearised plasmid DNA

To prevent self-ligation of linearised plasmid, and hence a high background of vector compared to clones of interest, shrimp alkaline phosphatase (Roche) was used, as per manufacturer's instructions.

2.3.9b Ligation of DNA fragments into plasmid DNA vectors

Ligation of DNA and vector with compatible overhanging DNA ends was performed using the Ready-to-go™ ligation tubes (Amersham-Pharmacia). Briefly, the insert and plasmid DNA were added to the ligation tubes at a ratio of 3:1 ends. The amount of insert required depended on its size, and the following formula was used to work out how much insert was required for ligation to 100ng of plasmid:

$$\begin{aligned} &[(100\text{ng of vector} \times \text{kb size of insert}) / \text{kb size of vector}] \times 3 \\ &= \text{ng of insert} \end{aligned}$$

Blunt ended inserts were either generated by restricting the DNA with enzymes that produce blunt ends, or by Klenow (DNA polymerase I large fragment) treatment (Roche), or T4 DNA polymerase treatment (Roche). Klenow treatment (Anderson, S 1980) and T4 DNA polymerase treatment (Challberg, M.D. and Englund, P.T. 1980) were carried out as per manufacturer's instruction. PCR fragments were cloned using the perfectly blunt cloning kit, (Novagen). Briefly, 0.05pmol of insert was added to 5µl of end conversion mix and made up to 10µl with double distilled water. The reaction was incubated at 22°C for 15 minutes, then 1µl of blunt vector (pstBLUE-1) and 1µl of T4 DNA ligase was added to the end conversion reaction and incubated at 22°C for 15 minutes.

2.3.9c Transformation of Competent Cells

Chemically competent cells were thawed on ice and gently re-suspended, before adding 2µl of ligation reaction. The cells were incubated on ice for 15 minutes and heat shock treated at 42°C for 30 seconds before being returned

to ice for two minutes. A 250µl volume of SOC medium (GibcoBRL) was added, and the cells were incubated at 37°C with shaking for 30 minutes. Between 50µl and 250µl were plated onto Luria-Bertani-agar (LB-agar) plates, and incubated overnight at 37°C.

Transformation efficiency was calculated by transforming a known quantity of a purified supercoiled plasmid DNA. The number of colonies formed (CFU) were counted and the transformation efficiency was calculated using the following formula:

$$\frac{\text{Number of colony forming units(CFU)} \times 10^3 \text{ ng} \times \text{final dilution plated}}{\text{ng of supercoiled vector plated} \times 1 \mu\text{g}} = \text{transformation efficiency (cfu/}\mu\text{g)}$$

2.3.9d Preparation of LB-Agar Plates

LB-Agar (Anachem) was prepared as per manufacturer's instructions, and autoclaved. The LB-Agar was melted in a microwave, and allowed to cool to 60°C before addition of ampicillin (100µg/ml) or kanamycin (30µg/ml) depending on the plasmid selection marker. The plates were prepared by pouring 30ml of the LB-Agar into 7.5cm petri dishes and were left to set. For blue/white colour selection 100µl of 20mg/ml X-Gal, (Roche) and 100µl 0.1M IPTG (Roche) were spread over the plates and allowed to soak into the agar, before transformations were plated out.

2.3.10 Propagation of DNA Clones and Their Purification

2.3.10a Small Scale Purification of Plasmid DNA

Colonies were picked from LB-agar plates and transferred to 5ml of LB-broth containing ampicillin or kanamycin. These were grown overnight at 37°C with vigorous shaking. The DNA was extracted using either the Mobio

mini-prep kit (Cambio), Mini-prep kit (Sigma), or jetSTAR™ mini-prep kit (AMS Biotechnology) according to manufacturers' protocol. Typically, a yield of 0.5µg to 20µg was obtained depending on the mini-preparation kit used. Positive plasmid clones were identified by restriction digest, and by automatic DNA sequencing (refer to section 2.3.11c). These positives were stored as glycerol stocks of bacterial cultures.

2.3.10b Glycerol stocks of bacterial cultures

Glycerol stocks were made by adding 500µl of bacterial culture, grown to stationary phase, to 500µl of freezing mix (40% glycerol, 10mM MgSO₄ 0.5% NaCl). These were inverted to mix, and stored in the -70°C freezer.

2.3.10c Large scale purification of plasmid DNA

2.3.10ci Qiagen column method

This method was used to isolate >1mg of plasmid DNA rapidly. 100ml of LB-broth with either ampicillin or kanamycin, was seeded from the mini-preparation and allowed to grow overnight at 37°C with shaking. The Qiagen protocol was followed, with a final phenol chloroform step added after propan-2-ol precipitation of the DNA.

2.3.10cii Equilibrium Centrifugation of Plasmid DNA in CsCl ethidium bromide gradients

This method was used to generate about 3mg of high quality, supercoiled DNA to be used in applications such as transfections. It involves DNA extraction by the alkaline lysis method (Birnboim, H.C., Doly, J. 1979) and subsequent plasmid purification by centrifugation.

A small-scale bacterial culture, was seeded into 500ml of LB-broth (Anachem, prepared as per manufacturer's instructions), with suitable antibiotic added, and grown overnight at 37°C with shaking. The bacterial culture was harvested into two 250ml centrifuge bottles, and centrifuged at 6000rpm in the J2-21 Beckman centrifuge, with the JA-14 rotor at 4°C for 10 minutes. The broth was decanted into 5-10% Presept (Johnson & Johnson) and the bacterial pellets re-suspended in 36ml of solution P1 (50mM Glucose, 25mM Tris-HCl pH 8, 10mM EDTA). Addition of 36ml of solution P2 (0.2M NaOH, 1% w/v SDS), and incubation at room temperature for 5 minutes, was followed by addition of 36ml of chilled solution P3 (3M KAc pH 4.8). This was incubated on ice for 15 minutes, then centrifuged at 7000rpm, at 4°C for 15 minutes. The supernatant was filtered through Miracloth (Camlab) into clean centrifuge bottles and 67ml of propan-2-ol (0.7 volumes) was added. The DNA was pelleted by centrifugation at 7000rpm at 4°C for 15 minutes, the pellet re-suspended in 4ml TE, and transferred to 15ml culture tubes (Elkay).

The plasmid DNA was phenol chloroform extracted, and ethanol precipitated. The DNA was centrifuged using a JA-20.1 rotor at 8000rpm at 4°C for 5 minutes and washed with 70% ethanol. The centrifugation step was repeated and the ethanol removed. The DNA pellet was air-dried, and re-suspended in 2ml of TE.

To prepare the CsCl gradient, 11.2ml distilled water was pipetted into a 15ml orange screw cap (Costar) tube, and the meniscus was marked on the tube. The water was discarded, and 9.46g of CsCl (Roche) was added with all the plasmid DNA and 500µl of 10mg/ml ethidium bromide was added. The volume was made up to the meniscus mark with TE and pipetted into two

Sorvall ultracentrifugation tubes. The tubes were balanced, and centrifugation was carried out at 50000rpm in the Sorvall UltraPro 80 in a TV 1665 upright rotor (Sorvall) at 20°C for 16 hours.

The bands of DNA were visualised using long wavelength (302nm) UV light. An 18 gauge needle attached to a 2ml syringe, was used to draw out the plasmid band which was transferred to a 15ml screw cap tube (Costar). Ethidium bromide was removed by adding one volume of CsCl saturated propan-2-ol, the tube was shaken and the upper phase was removed and discarded. This was repeated until both phases were clear and colourless. The DNA was ethanol precipitated at -20°C for 1 hour.

The tubes were centrifuged at 8000rpm for 10 minutes, and the supernatant removed. The pellet was washed with 500µl 70% ethanol and re-centrifuged. The ethanol was removed, and the pellet allowed to air-dry before re-suspending in 500µl TE and transferred to a 1.5ml eppendorf tube. The DNA was phenol chloroform extracted, and transferred to a fresh eppendorf tube. The DNA was ethanol precipitated centrifuged at 13000rpm, the supernatant was discarded, and the pellet allowed to air-dry. The final pellet was re-suspended in 1ml of low TE (10mM Tris-HCl pH 8, 0.01mM EDTA) and quantified.

2.3.11 DNA sequencing

2.3.11a Manual DNA sequencing

Manual sequencing was performed using the Pharmacia T7 sequencing kit, and ³⁵S α dATP (1000Ci/mmol) (Amersham-Pharmacia). Briefly 2µg of CsCl purified DNA, or 8µl of mini-prep DNA was used. 2µl 2M NaOH (freshly made up) was added, and the volume increased to 10µl with water. The

sample was ethanol precipitated with 6µl 3M sodium acetate pH 5.4, 60µl ethanol, and 7µl of water and reconstituted in 10µl of water before annealing with 1µl of 40µg/µl of primer, 2µl of annealing buffer, and 1µl water at 37°C for 20 minutes, and allowed to cool back to room temperature. Tubes were labelled A, C, G, T, and 2.5µl of the relevant dideoxy terminator was added to the relevant tube. The enzyme premix was prepared (for each tube, 2µl enzyme dilution buffer, 0.35µl T7 DNA polymerase, 1µl water, 3µl labelling mix A, and 1µl ³⁵S α dATP was required), and kept on ice. A 6µl aliquot of the premix was added to the annealed template and primer and incubated at room temperature for 5 minutes. The dideoxy sequencing terminators were warmed to 37°C, before 4.5µl of the labelling reaction was added to each of the sequence terminators, and incubated for 5 minutes at 37°C. Stop solution, (5µl) was added and the sample stored on ice until required. Just before loading, the samples were heated to 100°C, and 2µl were loaded onto a 6% / 7M urea denaturing acrylamide (19:1 acrylamide:bis-acrylamide) gel.

2.3.11b Denaturing Acrylamide gel electrophoresis

The sequencing plates (Bio-Rad) were cleaned thoroughly with Decon 90, distilled water, and polished with ethanol. The back plate was siliconised with Glass Shield (Genomymx). A 6% / 7M urea denaturing (19:1 acrylamide:bis-acrylamide) acrylamide gel was prepared, polymerised with 300µl 10% ammonium persulphate (APS) and 30µl TEMED, and poured. The samples were heated to 80°C before 2µl was loaded and electrophoresed for 2 hours at 60W. The acrylamide gel was fixed in 10% acetic acid and dried on

a gel drier, at 80°C for 1 hour. The gel was exposed to Kodak AR autoradiographic film overnight.

2.3.11c Automatic DNA sequencing

This method was performed using the ABI BigDye™ terminator kit. The PCR reaction was set up according to the user protocol, and was carried out using 25 cycles of denaturation at 96°C for 30 seconds, followed by annealing at 50°C for 15 seconds, and extension at 60°C for 4 minutes, finishing with indefinite cooling at 4°C. To precipitate the DNA, 16µl of water and 64µl of 95% ethanol were added. After 15 minutes at room temperature, the samples were centrifuged at 13000 rpm for 20 minutes, the supernatant removed and the DNA pellet air dried, then reconstituted in 4µl loading buffer (5:1 deionised formamide, 25mM EDTA pH8). A 12%/8M urea denaturing (19:1 acrylamide:bis-scrylamide) acrylamide gel was prepared and poured. The sample was heated to 100°C before loading onto the gel in alternate lanes and electrophoresed on an ABI 373A sequencer.

2.3.12 Southern blotting of genomic DNA

2.3.12a Restriction digest of genomic DNA and agarose gel electrophoresis

*Hind*III and *Bam*HI (Roche) was used to digest 20µl of mouse genomic DNA overnight at 37°C in a total digest volume of 200µl. Approximately 10µg of this restricted DNA was loaded onto a 14cm 0.8% 1xTAE agarose gel, with 10µl of 1µg/µl of bacteriophage lambda DNA restricted with *Hind*III as a size marker. The gel was electrophoresed at 2V/cm overnight.

2.3.12b Depurination, and denaturing of DNA in an agarose gel

The gel was stained with 1x TAE running buffer containing 0.5µg/µl ethidium bromide for 10 minutes, and destained for 20 minutes in fresh 1x TAE running buffer. To aid transfer of DNA onto the Hybond-N nylon membrane (Amersham-Pharmacia), the DNA was treated with 0.2N hydrochloric acid for 10 minutes, then immersed in denaturing solution (1.5M NaCl, 0.5N NaOH) for 45 minutes, rinsed with distilled water and neutralised with neutralisation solution (1.5M NaCl, 1M Tris pH 7.4) for 30 minutes, this was discarded and fresh solution added for a further 15 minutes.

2.3.12c Transfer of nucleic acid to a nylon membrane

A sheet of Hybond-N (Amersham-Pharmacia) and 3 pieces of 3MM (Shleicher and Schuel) were cut to the size of the gel. A further two pieces of 3MM were cut length-ways to act as a wick. A glass dish was filled to half way with 20x SSC (0.3M sodium citrate, 3M NaCl) and a platform was made over this reservoir. The long pieces of 3MM were soaked in 20xSSC and placed on the platform, with their ends in the reservoir. The gel was placed on the wick and the gel edges were surrounded with Saran wrap. The Hybond-N was pre-soaked in 20x SSC and was placed on top of the gel, followed by the pre-soaked pieces of 3MM. A 5cm stack of absorbent towels were placed on top of the 3MM, with a glass plate and a weight balanced on the stack. During the first hour of transfer, the paper towels were changed every 15 minutes, then the transfer was left to proceed overnight.

2.3.12d Cross-linking the DNA to the membrane and storage

A piece of 3MM was cut slightly larger than the gel and soaked in 20x SSC and placed on a bed of clingfilm. The transfer apparatus was dismantled. Before removing the membrane from the gel, a small cut was taken from the right hand corner and the wells marked with a pencil. The membrane was removed from the gel, inverted 180° and placed on the wet 3MM, so that the DNA side faced upwards. The membrane was cross-linked using an UV cross-linker (Spectrolinker™ XL-1000, Spectronic corporation), washed gently in 20x SSC, and the excess liquid removed. The membrane was wrapped in Saran wrap, and stored at 4°C until required.

2.3.13 Guidelines for working with RNA

All non-disposable glass was baked at 200°C and where possible, unopened disposable plastic-ware was used. Water was treated with 1/100 volume diethyl pyrocarbonate (DEPC, Sigma), left overnight, and autoclaved 3 times to remove all traces of DEPC. All solutions for RNA use were prepared using DEPC treated water.

2.3.14 RNA extraction

RNA from L β T2 cells was extracted using the protocol supplied with RNazol B (AMS Biotechnology) based on the method published by Chomczynski and Sacchi (1987). Briefly, the cells were washed twice with cold PBS and an appropriate volume of RNazol B was added. The mix was scraped using a rubber-policeman (Nalge Nunc) into either 1.5ml eppendorf tubes or 15ml blue cap culture tubes (Elkay), 0.2 volumes of chloroform was added, the

tubes were mixed vigorously and incubated on ice for 5 minutes. The samples were centrifuged at 13000 rpm for 15 minutes, the top phase was removed to a fresh tube, and an equal volume of propan-2-ol was added. The tubes were capped, inverted to mix, then incubated on ice for 15 minutes before being centrifuged at 13000 rpm at 4°C for 15 minutes. The pellet was washed in 75% ethanol, centrifuged at 8000 rpm, and reconstituted in an appropriate volume of DEPC water and spectrophotometrically quantified (refer to section 2.3.8). The sample was then either stored under ethanol or frozen, at -70°C.

2.3.15 Preparation of cultured cell lysate for ribonuclease protection assays

Cells were washed twice in cold PBS before 1ml per 1×10^7 cells of Direct Protect™ Lysis buffer (Ambion, AMS) was added to the cells, and scraped with a rubber policeman into appropriate sized tubes. The lysate was then drawn up and down a thin gauge needle to shear the DNA and stored at -70°C until required.

2.3.16 Reverse Transcriptase Polymerase Chain Reaction

First strand cDNAs were generated using the first strand cDNA synthesis kit (Amersham-Pharmacia) for subsequent differential display and PCR analysis. A 5µg aliquot of total RNA was pipetted into an eppendorf tube and brought up to a total volume of 8µl with DEPC treated water. The RNA was denatured at 65°C for 10 minutes, and chilled on ice. Into a fresh 1.5ml eppendorf tube, 5µl of bulk first strand mix was added with the heat-

denatured RNA, and 1 μ l of dithiothreitol (DTT), and dT₁₂VN primer (where V = A, G, C, T and N = A, G, C, 24 μ M, 8 μ M each of N). The sample was pipetted to mix, and incubated at 37°C for 1 hour. The subsequent cDNA was aliquoted as 2 μ l aliquots into labelled 0.5 μ l eppendorf tubes.

2.3.17 Northern blotting of total RNA

2.3.17a Agarose gel electrophoresis under denaturing conditions

Agarose, (1.5% w/v) was melted in 1xMOPS [20mM MOPS (3N-Morpholino propanesulfonic acid), 1mM EDTA pH8, 5mM sodium acetate pH 7] buffer. The gel was allowed to cool to 60°C, before 17ml of formaldehyde was added, per 100ml of gel.

The RNA was denatured at 65°C for 5 minutes in 38 μ l of denaturing buffer (25 μ l formamide, 5 μ l 10xMOPS buffer, 8 μ l formaldehyde) per 12 μ l RNA. This was snap cooled on ice, and 10x RNA loading dye solution added (50% (v/v) glycerol, 0.1mg/ml bromophenol blue). This was loaded onto the gel including a control lane of 5 μ g total RNA, and electrophoresed in 1xMOPS buffer at 1.5V/cm overnight.

2.3.17b Transfer of RNA to nylon membranes

The transfer apparatus was exactly the same as the one used for DNA transfer described in section 2.3.12c except, the nylon membrane and the 3 pieces of 3MM were soaked in 3x SSC. Before the nylon membrane was placed on the gel, the control lane was cut off, using a sterile scalpel. This was stained with 1xMOPS running buffer containing 0.5 μ g/ μ l ethidium bromide to visualise the ribosomal RNA bands of 18S and 28S rRNA. After

overnight transfer, the RNA was cross-linked to the membrane as before (section 2.3.12d), gently washed in 3x SSC, and stored in Saran wrap at 4°C.

2.3.17c Preparation of DNA probes

The DNA probes were digested from plasmid DNA, and separated by gel electrophoresis on a 2-4% low melting agarose gel. This was melted at 37°C, and a small aliquot was run on a standard agarose gel for quantification. The DNA was not extracted from the gel.

2.3.17d Radioactive labelling of DNA probes

DNA probes were radiolabelled using the Rediprime™ II random labelling kit (Amersham-Pharmacia), and these were hybridised to both Southern and Northern blots. Approximately 25-30ng of the DNA probe was made up to 45µl with TE, heated to 100°C for 5 minutes, cooled to 37°C and added to the Rediprime™ tube. Then 5µl of ³²P αdCTP (3000ci/mmol, Amersham-Pharmacia) was added, and incubated at 37°C for 30 minutes. A 2µl aliquot of the reaction was pipetted onto glass filter paper (GFP), washed three times in 5% TCA (tri-chloroacetic acid)/2mM PPi (pyrophosphate), and once in 100% ethanol. The GFP was placed in a scintillation vial and counted in a beta-counter (1450 Microbeta Trilux (Wallac)), using the Cherenkov counting protocol. The resultant figure was halved and the specific activity calculated; $\text{Cpm (value given by the counter/2)} \times \text{mass of DNA used} \times 3 = \text{specific activity}/\mu\text{g DNA}$

After pre-hybridising the membrane (see below), the radiolabelled DNA probe was denatured with 250µl 2M NaOH at 37°C for 5 minutes, then 700µl of water and 50µl of 0.05M EDTA were added.

2.3.17e Hybridisation of radiolabelled probe to membrane

The membrane was pre-hybridised for 10 minutes in hybridisation tubes (HB-OV-BM, Hybaid) with hybridisation buffer (0.5M NaPO₄ pH 7.2, 1mM EDTA pH 8, 7% SDS, 15% (v/v) formamide, 1% (w/v) BSA) that was pre-heated to 65°C. This buffer was decanted, and fresh buffer added with the radiolabelled probe. The hybridisation was rotated overnight at 65°C. After hybridisation, the membrane was washed in pre-heated wash solution (2xSSC/0.1%SDS) 3x for 20 minutes. The membrane was then removed and wrapped in Saran wrap.

2.3.17f Detection of the hybridised probe

The membrane was fixed in a Phosphoimage cassette and exposed for 2 to 7 days. The image was then scanned using a Phosphoimager (Storm 860, Molecular Dynamics).

2.3.17g Stripping Northern blots

To enable the nylon membrane to be re-probed (e.g. 18S, for quantification), it was bathed in boiling 0.1% SDS solution. This was allowed to cool, with gentle agitation, back to room temperature. The membrane was re-exposed in a phosphoimage cassette, to ensure that all the hybridising probe had been removed.

2.3.18 Ribonuclease Protection Assays

2.3.18a Radiolabelling *in vitro* transcribed RNA

The template DNA was transcribed in the anti-sense orientation, usually with T7 RNA polymerase, and in general, did not exceed 350bp in length. MAXIscript™ Invitro transcription kit (AMS, Ambion) was used to make the RNA probes. Template was linearised, phenol chloroform extracted, ethanol precipitated and re-constituted in DEPC treated water at a concentration of 1µg/µl. The reaction was set up at room temperature, with enough DEPC treated water to make a final volume of 20µl. Then, 2µl 10x transcription buffer, 1µl 10mM NTPs (excluding UTP), 1µg linearised DNA, 2.5µl ³²P γ UTP (NEN, 800Ci/mmol), and 2µl T7 or SP6 polymerase was added. The control DNA template was supplied with the kit, and was either glyceraldehyde-6-phosphate dehydrogenase (GAPDH), or 18s ribosomal RNA (18s). The reaction was modified to include, 1µl of cold 1mM, or 10mM UTP for the GAPDH, and 18s reactions respectively and 1.25µl of the radionucleotide was added to the GAPDH reaction, and 1µl of a 1:40 dilution of the radionucleotide was added to the 18S reaction. All reactions were incubated at 37°C for 1 hour, before addition of 1µl of RNase free DNaseI, and the incubation continued at 37°C for 15 minutes. The reaction was stopped by the addition of 20µl of RNase free formamide dye. This was heated to 90°C for 2 minutes, and loaded onto a 5% acrylamide (19:1 acrylamide:bis-acrylamide), 8M urea gel. The gel was electrophoresed at 12.5V/cm for 1.5 hours using a Protean II xi cell electrophoresis tank (BioRad). The radio-labelled bands were cut out of the gel, and the gel fragment was transferred to 300µl of RNA elution buffer (0.5M NH₄OAC, 1mM EDTA pH 8, 0.1% SDS) and incubated overnight at 37°C. The 100bp ladder (supplied in the kit) was

transcribed and radio-labelled using the same reaction conditions as the GAPDH probe, however it was purified using a Chromaspin 10 column (Clontech) as per manufacturers instructions.

After incubation overnight, an equal volume of Tris-HCl pH 8 saturated phenol was added, the tubes were vortexed and centrifuged at 13000 rpm for 3 minutes. The top phase was removed to a fresh tube, and RNase free 100% ethanol, with 1µl of yeast tRNA (5µg/µl AMS, Ambion) was added. The RNA probes were precipitated at -20°C for 30 minutes, centrifuged at 13000 rpm for 5 minutes, the ethanol decanted, and the pellet washed with 80% ethanol. The pellet was re-suspended in 50-100µl Direct Protect™ lysis buffer, then 2µl was pipetted onto GFP, and Cherenkov counted on a betacounter (Wallac) as already described (section 2.3.17d). The cpm values were used to calculate the amount of probe required for the hybridisation.

2.3.18b RNase Protection Assay

The final reaction volume was 50µl and the hybridisations were set up as follows: test probes were added at 1×10^5 cpm/tube; and the control probes GAPDH and 18s were added at 6×10^4 cpm/tube and 1×10^4 cpm respectively. LβT2 cell lysate was added and the volume made up to 50µl with Direct Protect™ lysis buffer. The hybridisations were incubated overnight at 37°C. The next day, a RNase master mix was prepared (10µl RNase cocktail, 50µl 10x RNase digestion buffer, and 440µl water) and added to each hybridisation. This was inverted and incubated at 37°C for 30 minutes, then 20µl of 10% (w/v) sarcosine and 10µl of 20mg/ml proteinase K were added. The tubes were vortexed, and incubated at 37°C for 30 minutes. The RNA

was precipitated by adding 500 μ l of propan-2-ol and incubated at -20°C for 30 minutes. The samples were centrifuged at 13000rpm for 15 minutes, the supernatant decanted, and the tubes re-spun for a further 4 minutes. The excess supernatant was pipetted off, and 10 μ l of formamide dye was added. The pellets were re-suspended by vortexing, heated to 100°C for 2 minutes, and loaded onto a 5% acrylamide (19:1 acrylamide : bis-acrylamide)/8M urea gel. In addition, 1000cpm each of the unhybridised RNA probes, and 1000cpm of marker in 10 μ l of dye were also loaded onto the gel. The gel was electrophoresed at 12.5V/cm for 3 hours, fixed in 10% (v/v) acetic acid, transferred onto 3MM paper and dried at 80°C on a gel drier. The gel was exposed to a Phosphor screen for over 48 hours, and scanned into a Phosphoimager (Molecular Dynamics). The bands were quantified using the ImageQuant (Molecular Dynamics) and Excel (Microsoft) packages.

2.4 Mammalian Cell Culture

2.4.1 General notes

Dulbecco's Modified Eagle's Medium (DMEM, Sigma) which contained 10% (v/v) Fetal Calf Serum (FCS, Sigma) and 1% (v/v) Penicillin/Streptomycin (Pen/Strep, Sigma) was used, and will hereafter be described as DMEM. All solutions used were pre-heated to 37°C.

2.4.2 Cell lines used

The cell lines used, were HeLa cells (ECACC), L β T2 cells and α T3-1 cells. The L β T2 (Turgeon et al 1996) and α T3-1 (Wendle et al 1990) transformed cell lines were both derived by *in vivo* transformation and are of gonadotroph

lineage. The α T3-1 cells correspond to an earlier developmental age mouse pituitary embryonic age (e12-13.5) than L β T2 cells (e16.5). Therefore, α T3-1 cells only synthesise and secrete α GSU, whereas L β T2 cells can synthesise and secrete α GSU and both LH and FSH β -subunits (Turgeon et al 1996, Low et al 2000) and both cell-lines are responsive to GnRH.

2.4.3 Coating of culture flasks with Matrigel

All cell culture flasks (Costar), and 12 well plates (Costar/Nalgene) were coated with a 1/30 dilution of a basement membrane matrix (Matrigel™ Becton Dickinson Labware). The Matrigel™ was thawed overnight on ice, as the matrigel solidifies above 4°C, and 200 μ l were aliquoted into pre-chilled cryotubes (Nalge Nunc) and stored at -20°C. The Matrigel™ was used at a 1/30 dilution, so an aliquot was thawed on ice for 2 hours, and added to 5.8ml of cold phosphate buffered saline (PBS, Sigma) in a 15ml blue capped culture tube (Elkay), then aliquoted into 2.5ml aliquots, and stored in 5ml culture tubes at -20°C. To coat flasks, Matrigel™ was pipetted onto the base of the flasks or plates to be used, ensuring the whole area was covered, excess Matrigel™ was removed, and the flasks and/or plates were allowed to air-dry.

2.4.4 Resuscitation of Cell lines

Cells were removed from liquid nitrogen, and placed on ice. They were thawed by adding 500 μ l of pre-warmed DMEM (Sigma), then transferred into a pre-coated 25cm² culture flask (Costar) containing 4mls of DMEM. Resuscitated HeLa cells were transferred into flasks that had not been coated

with Matrigel™. The cells were incubated at 37°C in a humidified, 5% CO₂ atmosphere and the media was changed every 3-4 days.

2.4.5 Passaging of Cell lines

LβT2 and αT3-1 cells were passaged once weekly. The DMEM was removed, then the cells were washed with 2 changes of 2.5ml of PBS. Trypsin (Sigma) was diluted 1/10 with PBS and 500μl (for a 25cm² flask) or 1.5ml (for a 75cm² flask) was added before the flasks were returned to the incubator and left for 1-2 minutes. After the cells detached from the flask, the trypsin was inhibited by the addition of 4.5ml (25cm² flask) or 14.5ml (75cm²) of DMEM. The cells were dispersed, and LβT2 cells were then split 1:4 into 25cm² flasks, and 100000cells/ml into 12 well plates. αT3-1 cells were split 150000cells/ml into 25cm² flasks, and 100000cells/ml into 12 well plates. HeLa cells were split 100000cells/ml using 1x trypsin into non-Matrigel™ coated wells and flasks.

2.4.6 Estimation of Cell number

Cell number was estimated using a hemacytometer (Weber Scientific International Ltd). After the trypsin was inactivated, 10μl of cells was removed into 100μl of DMEM, and transferred to the hemacytometer. The cells were counted, and an average taken, this was the cell number x 10⁵ per ml. The cells were then diluted accordingly.

2.4.7 Long Term Storage of Cell lines

Cells were stored under liquid nitrogen and were prepared as follows. The LβT2 cells and αT3-1 cells were grown to 80% confluency before they were

trypsinised as previously described (section 2.4.5) and the volume made up to 5ml (for 25cm² flasks) or 10ml (for 75cm² flasks) with DMEM before spinning at 1000rpm (Econospin, Sorvall) for 5 minutes. The media was decanted, and the cells gently re-suspended in either 0.5ml or 1.25ml of FCS per 25cm² or 75cm² flask respectively. The cells were then combined before 500µl was aliquoted into pre-chilled, labelled cryotubes, and 500µl of 20% (v/v) of dimethyl sulfoxide (DMSO, Sigma) diluted in FCS was added. The vials were mixed, and transferred into a cryo 1°C freezing container (Nalgene) and stored at -70°C overnight, before they were transferred to liquid nitrogen.

2.4.8 GnRH treatment of cells

GnRH (Peninsula) was dissolved in PBS at a concentration of 100µM, 500µl aliquots were made, and stored at -20°C. The stock was diluted by addition of 1/100 volume of GnRH to DMEM, and mixed thoroughly. To pulse cells with GnRH, the media was removed from the cells and the DMEM+GnRH was added. The cells were incubated at 37°C for 15 minutes, before the DMEM+GnRH was removed, and replaced with normal DMEM. The cells were harvested for RNA as previously described (see section 2.3.14). For transiently transfected cells, 1ml of GnRH+DMEM was added to each of the wells, but not removed. The cells were harvested 6 hours post GnRH treatment, as described below.

2.4.9 Transient transfection of DNA into *in vitro* cultured cells

The cells were plated into 12 well plates (Costar/Nunc) 24 hours prior to use. Fugene[™] (Roche) and GenePorter[™] (GTS), which are cationic liposome-mediated techniques (Felgner, P.L. 1987), were used to transfect plasmid DNA into the cells, and the total plasmid DNA transfected into each well was 2 μ g. The plasmid carrying the gene promoter/reporter construct to plasmid DNA ratio was always 6:1, so pBluescript (Stratagene) was added when necessary to make up the final mass of transfected DNA to 2 μ g per well. A β -galactosidase reporter construct, or a chloramphenicol acetyl transferase (CAT) reporter construct was co-transfected at 100ng per well as a control for transfection efficiency. Each transfection condition was carried out in triplicate and each experiment was repeated three times.

2.4.9a Transient transfection of α T3-1 cells and HeLa cells using GenePorter[™]

α T3-1 cells were transfected using GenePorter[™] (GTS) following the manufacturer's protocol. Cells were plated in 12 well plates 24 hours prior to use, the media was removed, and the cells were incubated with 500 μ l of Optimem (GibcoBRL) for 2 hours. The GenePorter[™] was prepared by adding 5 μ l per 1 μ g plasmid DNA. This mix (2 μ g DNA/well) was transfected and incubated for 4 hours in the CO₂ incubator, then the Optimem was removed, and DMEM added. The cells were returned to the incubator for 48 hours.

2.4.9b Transient transfection of L β T2 cells using Fugene[™]

Fugene™ (Roche) was used to transfect plasmid DNA into LβT2 cells. DNA (2μg), was added to the bottom of a 5ml culture tube (Elkay). Optimem was pipetted, to give a final transfection volume of 100μl per well, into a separate 5ml culture tube, Fugene™ (3μl/1μg DNA) was then added to the Optimem and this mix was transferred onto the DNA drop-wise. This was mixed and incubated at room temperature for at least 15 minutes. Then 100μl of transfection mix, was added drop-wise to each well, the plate was swirled gently to mix, and the cells were returned to the incubator for 48 hours. Cells were harvested, as described below, 6 hours post GnRH treatment.

2.4.10 Luciferase/β-galactosidase chemiluminescent reporter gene assay

These assays were carried out using the Dual Light™ kit (Tropix). The DMEM was removed from the wells, and the cells washed 3 times with cold PBS before adding 250μl of lysis buffer with protease inhibitors (Roche). The cells were shaken on a plate shaker for 20 minutes before being transferred to 1.5ml eppendorf tubes. The tubes were centrifuged at 13000rpm for 2 minutes to pellet the cellular debris. Duplicate 10μl aliquots of each sample were pipetted into two wells of a white 96 well plate (Nunc) and the luciferase gene activity was determined by the amount of light produced, in an automatic injection microplate luminometer (LB 96V, Microlumet Plus, EG+G Berthold). The plates were incubated for another 30 – 60 minutes and measured for the β-galactosidase activity. Luciferase light units were corrected by the relative amount of β-galactosidase activity in each sample.

2.4.11 β -galactosidase chemiluminescent reporter gene assay

Gene activity was assayed using the β -galactosidase reporter gene assay kit (Roche). The cells were washed in PBS and lysed using the buffer supplied. Duplicate 50 μ l aliquots were pipetted into 2 wells of a 96 well plate, and 100 μ l of substrate reagent was added manually. This was incubated at room temperature for 1 hour, before β -galactosidase activity was measured by the injection of 50 μ l of initiation reagent on the LB 96V luminometer.

2.4.12 Chloramphenicol acetyltransferase (CAT) assay

Transfection efficiency was measured by pipetting 30 μ l of cell extract into 1.5ml eppendorf tubes, and adding 164 μ l of 0.25M Tris-HCl pH 7.8. Heating to 65°C for 10 minutes inactivated endogenous acetyltransferases, before 1 μ l of 14 C-Chloramphenicol (50 –62mCi/mmol), 5 μ l of 50mM Acetyl Coenzyme A and enough 250mM Tris.HCl pH 7.8 to increase the volume to 200 μ l was added and incubated at 37°C overnight. Next day, 600 μ l of ethyl acetate was added to each reaction, which was vortexed 3 times for ten seconds each before centrifuging for 2 minutes at 13000rpm and removing the top organic phase to a fresh tube. The tubes were dried at 45°C under a stream of nitrogen gas. Thin layer chromatography (TLC) plates (Camlab) were cut to make 2 plates of 20cm x 10cm and marked 1cm from the bottom edge and along this line, spot positions were marked 1.3cm apart. The chromatography solvent (chloroform:methanol at a ratio of 95:5) was freshly prepared, and allowed to equilibrate for 10 minutes in the chromatography tank.

The dried reactions were reconstituted in 20 μ l of ethyl acetate and spotted onto the marked TLC plates, 2 μ l at a time. The TLC plates were placed in the tank until the solvent front reached the top of the plate, then removed from the tank and allowed to air-dry before being wrapped in Saran wrap and exposed to a Phosphoimage screen for 1 week. The activity of CAT was measured by quantifying the two faster migrating bands (the modified mono- and di-acetylated forms of 14 C-chloramphenicol) and dividing this by the quantified, lower unmodified band of 14 C-chloramphenicol. The result was multiplied by 100, which gave the percentage conversion rate.

2.4.13 Protein Quantification

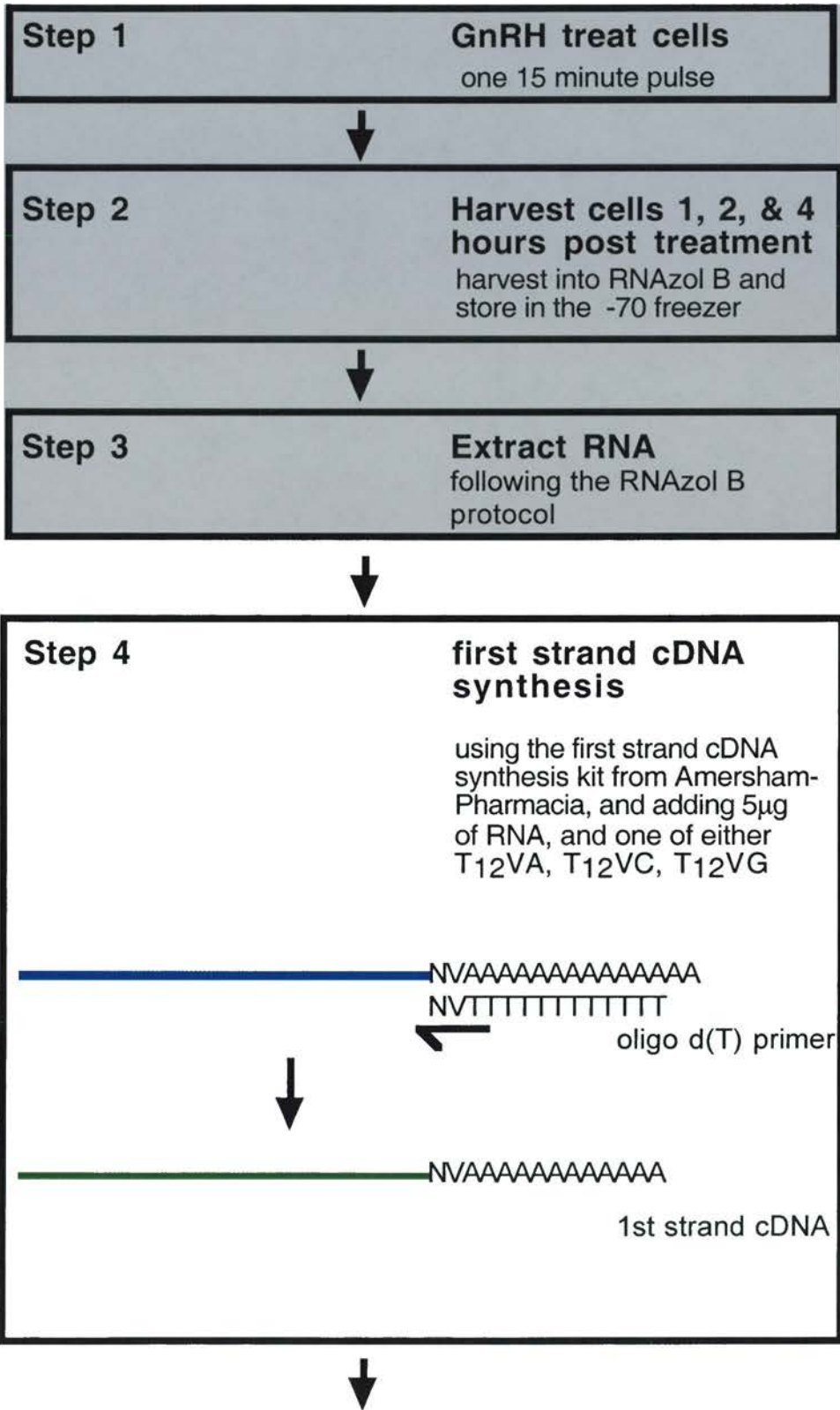
Protein concentration was determined using the Bradford protein assay (Bradford, M. 1976) reagent (BioRad). A standard curve using different concentrations (0 μ g/10 μ l, 0.5 μ g/10 μ l, 1 μ g/10 μ l, 5 μ g/10 μ l, and 10 μ g/10 μ l) of BSA was prepared for each assay and made up to 800 μ l with deionised water in 1.5ml eppendorf tubes. The protein concentration of each cell lysate was measured by pipetting 10 μ l of the sample into 790 μ l of deionised water, before 200 μ l of protein assay dye reagent (BioRad) was added to the water, the standard curve samples, and the samples to be measured. The reaction was mixed, and incubated at room temperature for 5 minutes, before measuring absorbance on a calibrated spectrophotometer (6150 UV/Vis Spectrophotometer, Jenway) at 595nm. The standard curve was plotted as a graph and the linear equation of the line determined. This equation was used to calculate the concentration of the protein from the absorbances.

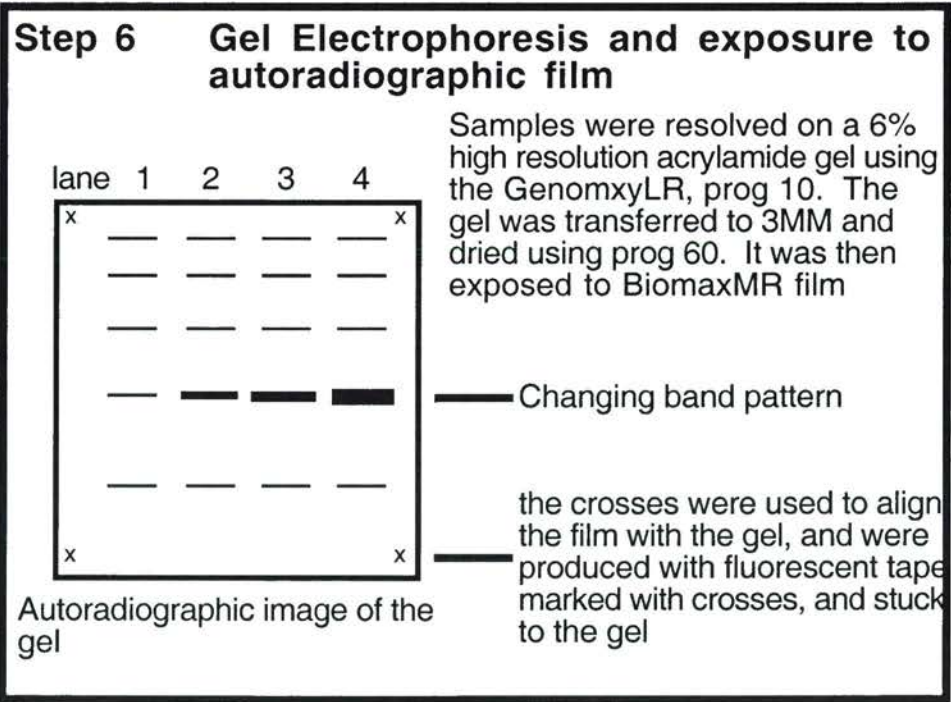
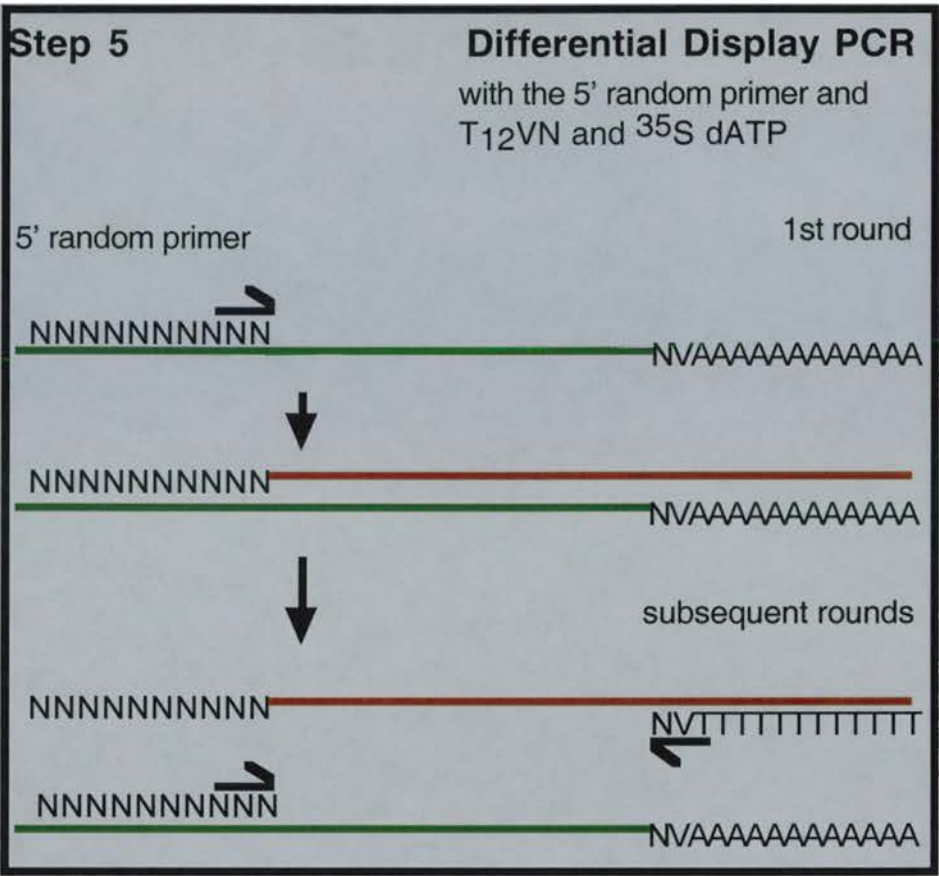
Chapter 2b: Differential Display Materials and Methods

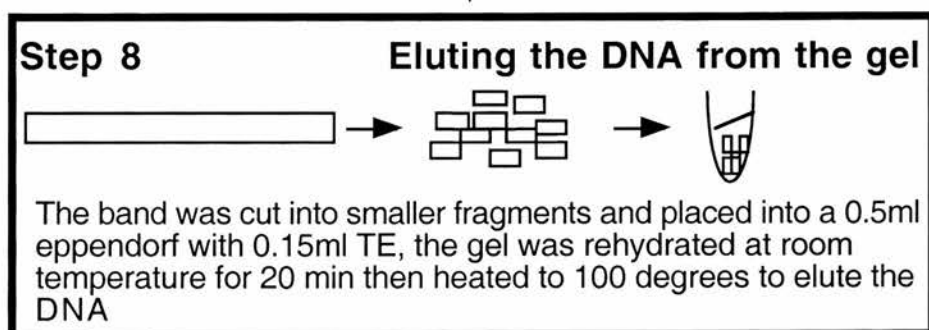
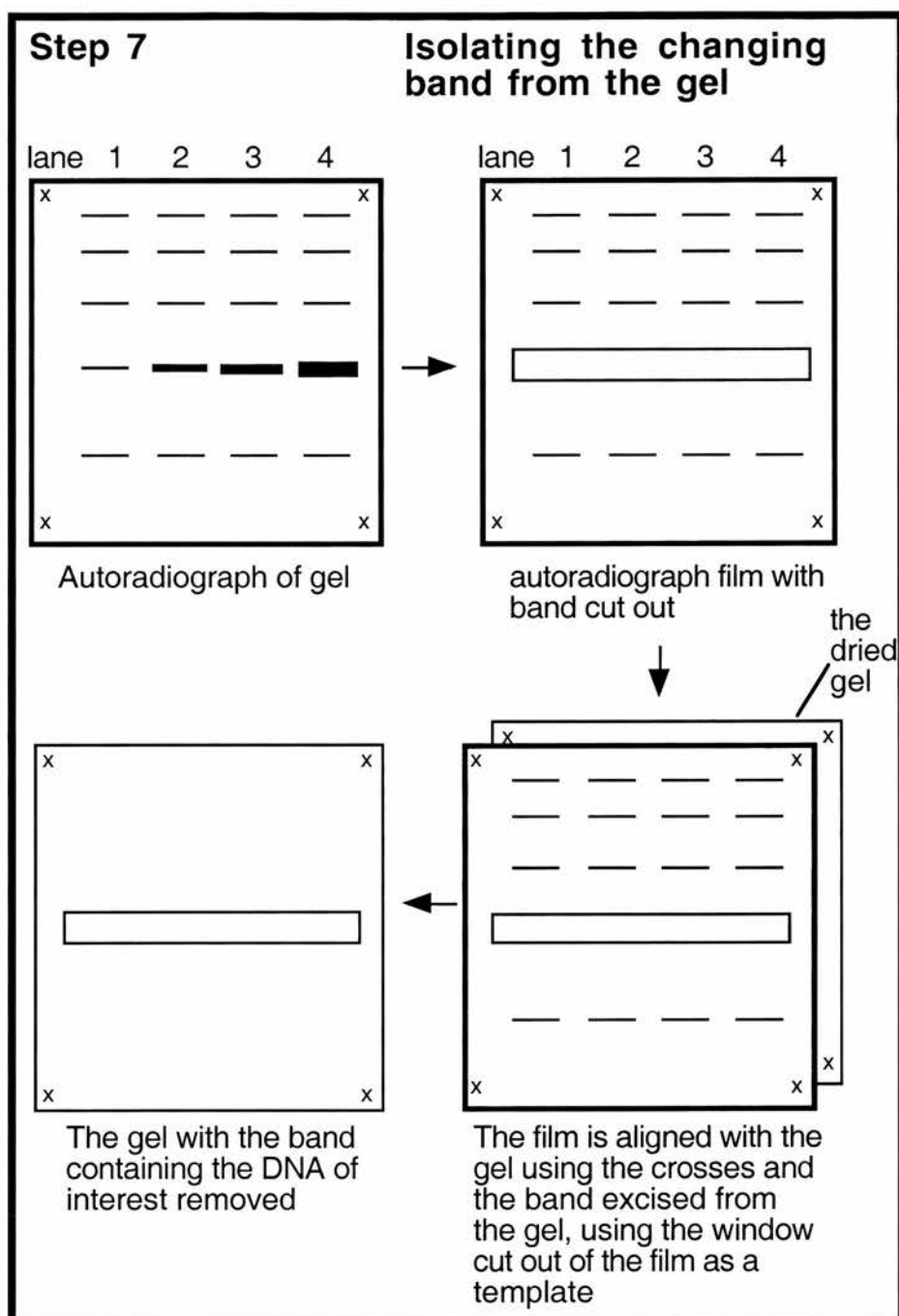
2.5.1 Introduction

Differential Display RT-PCR (DD-RT-PCR), developed by Liang and Pardee (Liang and Pardee 1992), is a method used to identify differentially expressed mRNAs, and a schematic of the major steps in the technique are shown in Fig. 2.5.1. DD-RT-PCR has a number of advantages over other methods of isolating differentially expressed transcripts (e.g. subtractive hybridisation), which include: (i) low quantities of starting material, as little as 200ng of total RNA, (ii) allows a direct visual comparison of different samples or treatments on the same gel, and (iii) high sensitivity. In addition, due to the variation in the downstream anchored primers (see Fig. 2.5.1, Step 4) the technique ensures amplification of all polyadenylated RNA, and since the technique is PCR based, low copy number transcripts are also amplified, so are included in the analysis. However, the major drawback to utilisation of this technique is false positives. False positives are transcripts that appear to be differentially expressed, but when double-checked are not. These can be artefactual bands on the gel (Fig. 2.5.1, Step 6) and are produced by non-standard RNA extraction and cDNA synthesis, or can be due to identically sized DNA fragments co-migrating with the band of interest. To minimise this, samples were handled in an identical manner, so RNA was isolated and cDNA was synthesised simultaneously. To minimise false positives due to identical sized DNA fragments, single stranded conformation polymorphism (SSCP) can be used (Miele, MacRae et al. 1998), which separates transcripts based on conformation, rather than size.

Fig. 2.5.1: Schematic representation of the DD-RT-PCR method







Step 9**Ethanol precipitation of eluted DNA**

The solution containing the DNA was transferred to a fresh eppendorf tube and the DNA precipitated with 3V 100% ethanol, 1/10V sodium acetate, 1µl of See-DNA and precipitated in the -70 freezer

**Step 10****PCR re-amplification of DNA isolated from the gel**

The DNA was pelleted by centrifugation, and re-constituted in 10µl low TE. This was amplified using AGS Gold Taq, the 5' random primers, the anchored 3' primers, in the DNA Engine

**Step 11****Cloning of PCR product**

The PCR product was cloned using the Perfectly Blunt cloning kit

**Step 12****Automatic DNA sequencing and identification**

The cloned DNA fragment was sequenced on an ABI 373A DNA sequencer, and the results were used in a BLAST search of the EMBL, and mouse EST databases

**Step 13****Confirmation of mRNA expression patterns**

The cloned PCR product was used as a radioactively labelled probe in Southern and Northern analyses, and as a template to in vitro transcribe a radioactively labelled probe for ribonuclease protection assays

DD-RT-PCR was used to analyse changes in gene expression in the L β T2 gonadotroph cell-line, that had been treated with GnRH. The differentially expressed transcripts, that were analysed, are presented in chapter three of this thesis. The next section briefly describes the DD-RT-PCR technique with the modifications that had to be included and is presented as a mini-results chapter.

2.5.2 Materials

Table 2.5: Components required for DD-RT-PCR

Reagents, kits and equipment	Supplier
40% Genomyx acrylamide gel	Beckman Coulter (UK)
$\alpha^{35}\text{S}$ dATP 1000Ci/mmol	Amersham-Pharmacia (UK)
RNazol B	AMS Biotechnology (UK)
First Strand cDNA synthesis kit	Amersham-Pharmacia (UK)
AmpliTaq [™] Taq Polymerase	Promega (UK)
AGS gold [™]	Hybaid (UK)
Pfu polymerase	Stratagene (UK)
High Fidelity Extensor Mix	AB Gene (UK)
KODAK BiomaxMR film 60cm x 33cm	Amersham-Pharmacia(UK)
See DNA	Amersham-Pharmacia (UK)
Oligonucleotides	Sigma-Genosys (UK)
Ready-to-go Ligation tubes	Amersham-Pharmacia (UK)

Perfectly blunt cloning kit	Novagen (UK)
Chromaspin columns	Clontech (UK)
UNOII PCR machine	Biometra
DNA Engine Gradient cyclor PTC-200	GRI (UK)
GenomylxLR DNA analyser	Beckman Coulter (UK)
³² P α dCTP (3000Ci/mmol)	Amersham-Pharmacia (UK)
2x MDE™	Flowgen (UK)

2.5.3 Method

2.5.3a Modified Differential Display RT-PCR (DD-RT-PCR)

A DNA marker was initially prepared by digesting pBluescript with *Sau3AI* (New England Biolabs) in a final concentration of 1µg/µl. The DNA was end labelled using Klenow (DNA Polymerase I large fragment, Roche) in the following reaction and incubated at room temperature for 30 minutes:

1µg of *Sau3AI* digested DNA
2µl 10x Klenow buffer
5µl ³²P α dCTP (3000Ci/mmol)(Amersham-Pharmacia)
1µl 2mM dNTP (without dCTP)(Amersham-Pharmacia)
2 units of Klenow

A Chromaspin 10 column (Clontech) was used to purify the marker following the manufacturer's instructions and a Cherenkov count was performed. An aliquot of the marker was diluted to 1000cpm/µl and 2000cpm, with 2µl 6x type 3 loading dye (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF) was loaded onto each gel.

2.5.3b Extraction of RNA

Briefly, L β T2 RNA was extracted from cells (Fig. 2.5.1, Steps 1–3) that had either been left untreated or treated with GnRH (refer to section 2.4.8), using the RNazol B protocol (see section 2.3.14).

2.5.3c Reverse transcription PCR

Total RNA was reverse transcribed into specific populations of cDNA using degenerate 3' oligo(dT) primers (Sigma-Genosys, Fig. 2.5.1, Step 4). The oligo(dT) primers were designed with 12 Thymidine bases and a combination of two random bases (AA, AG, AC, CA, CG, CC, GA, GG, GC; see appendix II) which would anchor the 3' primer to the poly A tail junction. In this way, all mRNAs within a sample of total RNA would be primed, and reverse transcribed.

The reverse transcription PCR (RT-PCR) was carried out as described in the previous section (see section 2.3.16). The oligo(dT) primers were pooled into three 24 μ M mixes; T₁₂VA, T₁₂VC, and T₁₂VG (see Appendix II), and used as the anchored 3' primers to fractionate the RNA into cDNA sub-populations.

2.5.3d Differential Display PCR

The cDNA was representative of the mRNA population of each treatment, and was used as a template in PCR reactions with the original 3' primers and an additional 5' random, 10mer primer (see appendix II for a full list of all primers used and Fig. 2.5.1, Step 5). Each cDNA sub-population was amplified using random 10mer 5' primers (see Appendix II), and the relevant anchored 3' primer mixes. Originally AmpliTaq™ (Perkin-Elmer) was the

preferred Taq polymerase used, however a direct comparison with AGS Gold™ (Hybaid) Taq polymerase, identified the latter as a more efficient enzyme for DD-RT-PCR (Fig. 2.5.2) and the protocol described below has been optimised for the AGS Gold™ Taq polymerase.

An aliquot of cDNA corresponding to each treatment, was diluted with 131µl of water. A 5µl aliquot of this was pipetted into labelled 0.5ml eppendorf tubes and 2µl of random primer (5µM) was added to each cDNA. A 1x master mix (see below) of the other PCR components was prepared on ice and 15µl was added to the DNA and random primer mix:

2µl 10x reaction buffer
2µl Enhancer
2.5µl 25mM MgCl₂
2µl 24µM T₁₂VN (N is A, G, or C)
2µl 20µM dNTP
1µl ³⁵S α dATP (1000Ci/ mmol)
0.3µl AGS Gold Taq
1.2µl water

The PCR was carried out under the following conditions: heating at 94°C for 2 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 40°C for 2 minutes, and extension at 72°C for 30 seconds. A final extension of 72°C for 5 minutes was performed, before the reactions were cooled indefinitely at 4°C, and 4µl of 6x type III loading dye was added.

2.5.3e Native Gel Electrophoresis

Changes in mRNA expression levels were visualised on autoradiographic film after electrophoresis on a native 6% polyacrylamide gel (Fig. 2.5.1, Step 6). A 4µl aliquot was loaded onto a 6% high resolution acrylamide gel (40% HR-1000 acrylamide, Genomix, Beckman), and electrophoresed on a GenomixLR DNA analyser (Beckman) at 2700V for 2 hours 15 minutes at

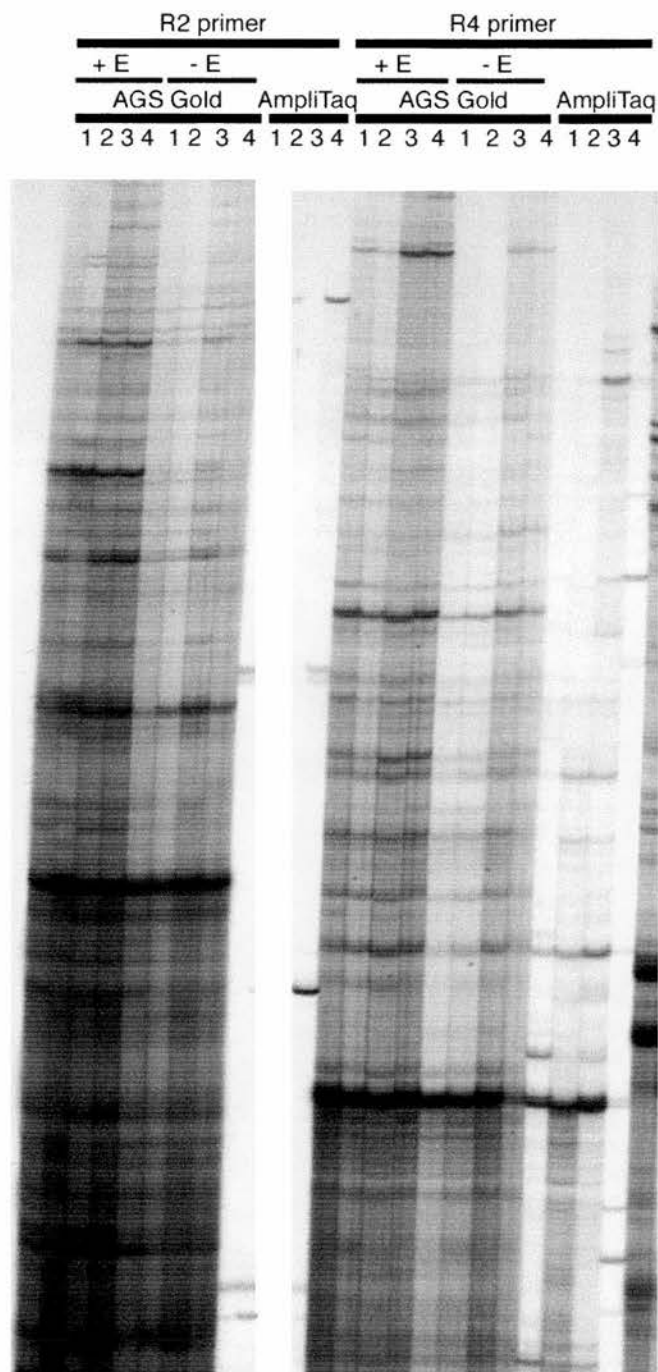


Fig. 2.5.2: DD-RT-PCR gel comparing the efficiency of AmpliTaq and AGS gold Taq polymerases.

An autoradiographic image of a DD-RT-PCR gel that has been exposed to a DD-RT-PCR dried gel for 6 hours. LβT2 cells were either untreated (lane 1), or treated with one 15 minute pulse of GnRH(lane 2), or treated with two 15 minute pulses 30 minutes apart (lane 3), or treated with two 15 minute pulses 90 minutes apart (lane 4). The DD-RT-PCR reactions with AGS Gold were carried out with and without Enhancer (+/-E), to identify if the Enhancer improved enzyme efficiency

50°C. The gel was transferred to 3MM paper, and dried in the Genomyx machine. Fluorescent marker tape (Tracker tape™, Amersham-Pharmacia) was used to align the film with the gel. The gel was exposed twice to BiomaxMR film, for 6 hours, and overnight.

2.5.3f Isolation of bands of interest from the gel

Changing band patterns were indicative of changes in mRNA expression and bands of interest were excised from the gel, using the film as a template and guide (Fig. 2.5.1, Step 7). The gel was then cut into smaller fragments, transferred into 0.5ml eppendorf tubes and re-hydrated in 150µl low TE at room temperature, for 20 minutes. The DNA was eluted at 100°C for 20 minutes, with occasional flicking, transferred to a fresh tube, and ethanol precipitated with 1µl See-DNA at -20°C for 1 hour. The sample was centrifuged at 13000rpm for 20 minutes to pellet the DNA and was re-suspended in 10µl of low TE (Fig. 2.5.1, Step 8 – 10).

2.5.3g SSCP analysis of differentially expressed transcripts

Originally, SSCP was performed on all transcripts isolated from differential display gels to ensure that the band of interest only contained one species of DNA, and to recapitulate the expression pattern seen on the differential display gel.

The band of interest was cut out of the differential display gel as individual lanes, and the DNA isolated as described above. The DNA from each lane was re-amplified using the high fidelity extensor Taq polymerase (AB Gene) as per manufacturer's guidelines, and one round of either 10 or 15 cycles of PCR, using the PCR conditions described below (section 2.5.3h). A 15µl

aliquot of the PCR reaction was taken, and 5 μ l of 10x denaturing dye (20mM EDTA pH8, 0.05% (w/v) bromophenol blue, made up in deionised formamide) was added. The sample was denatured at 95°C for 5 minutes and stored on an ice/water mix before being loaded onto a 0.5x MDE™ (12.5ml 2x MDE™ [Flowgen], 37.5ml 1xTBE) minigel (Mini Protean 3, BioRad). The samples were electrophoresed at 0.8mA/cm for 1.5 hours and the bands were visualised by silver staining. The gel was fixed (50% (v/v) methanol, 10% (v/v) acetic acid) for 30 minutes, and washed (10% (v/v) ethanol, 0.005% (v/v) acetic acid) with 2x 3 minute washes. A 0.1% (w/v) AgNO₃ solution was prepared, and added to the gel for 20 minutes. The gel was developed by decanting the AgNO₃ solution and adding developer (3g NaOH, 0.8ml formaldehyde, 200ml ddH₂O) until the bands were at the desired intensity. The reaction was stopped with 0.75% (w/v) sodium carbonate, the gel was wrapped in Saran wrap, and a photographic image was taken (Fig. 2.5.3g).

In general only two bands were visible, which corresponded to the two strands of a single transcript, and recapitulated the pattern seen on the differential display gel. However, it was concluded that SSCP did not confirm the pattern of expression directly, because the bands on the SSCP gel were a direct comparison to the bands on the differential display gel, not a confirmation of mRNA transcript levels. It was also difficult to interpret SSCP gels that showed more than two bands, since it was impossible to distinguish between multiple bands as a result of different species of DNA, or as a result of a single species of DNA with more than one conformation. Since SSCP did not confirm mRNA expression directly, nor did it definitely identify if a differential display band contained only one transcript, it was

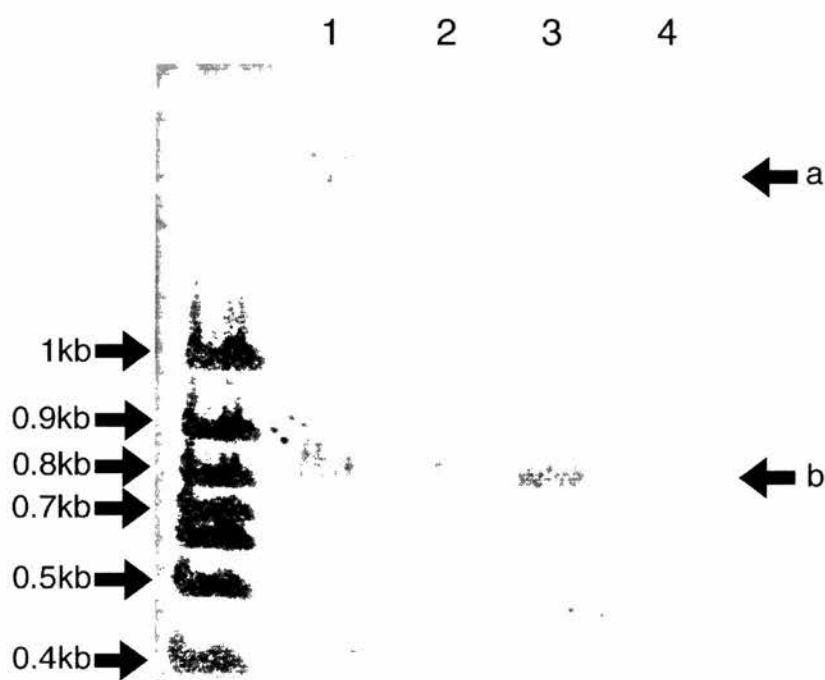


Fig. 2.5.3g: Recapitulating the differential display profile using SSCP.

Individual lanes were cut from the differential display gel and re-amplified using the high fidelity extensor PCR mix R1 and T₁₂VC primers, and 12 cycles of PCR. The DNA was denatured and electrophoresed on this 1xMDE gel and the bands visualised by silver staining. The pattern of the lower band (b) followed that of the R13c pattern (Fig 3.2.5a) and the upper bands (a) are probably un-denatured DNA. Size markers are included to provide an approximate estimation.

decided that SSCP would not be used, and the amplified DNA products would be cloned directly. It was also concluded that, since I used a transformed cell line, other contaminating mRNAs derived from other cell populations found in tissue samples would not be present, therefore the bands seen on a differential display gel were less likely to contain multiple transcripts, probably due to lower molecular complexity.

2.5.3h Re-amplification of DNA contained in the bands of interest

Once a transcript had been amplified it was cloned, sequenced, and identified (Fig. 2.5.1, Step 11 – 12). To produce PCR products with restriction sites on the end, *EcoRI* tagged 3' anchored primers and *XbaI* tagged 5' random primers were designed and used with AmpliTaq™ to amplify the isolated DNA fragments from DD-RT-PCR gels. Attempts to clone these products into *EcoRI* and *XbaI* restricted pBluescript were unsuccessful, possibly because the PCR reaction failed. To identify why these PCR fragments failed to clone, a control PCR experiment was carried out using 1 and 2 units of the AmpliTaq™ Taq polymerase, with α GSU specific primers, on sheep pituitary DNA (Fig. 2.5.3h[i]). The results showed that the Taq polymerase was not working efficiently. The next step was to test other Taq and Pfu polymerases, to identify the most efficacious for amplifying DD-RT-PCR isolated DNA fragments. PCR reactions were carried out using the unmodified primers at 20 μ M concentration, and either Pfu polymerase (Stratagene) or AmpliTaq™. Product could only be visualised with the Pfu polymerase after two rounds of 40 cycles of PCR (Fig. 2.5.3h[ii]) therefore AmpliTaq™ did not amplify the DNA eluted from the DD-RT-PCR gels as efficiently as Pfu polymerase. To test if the modified primers (restriction site

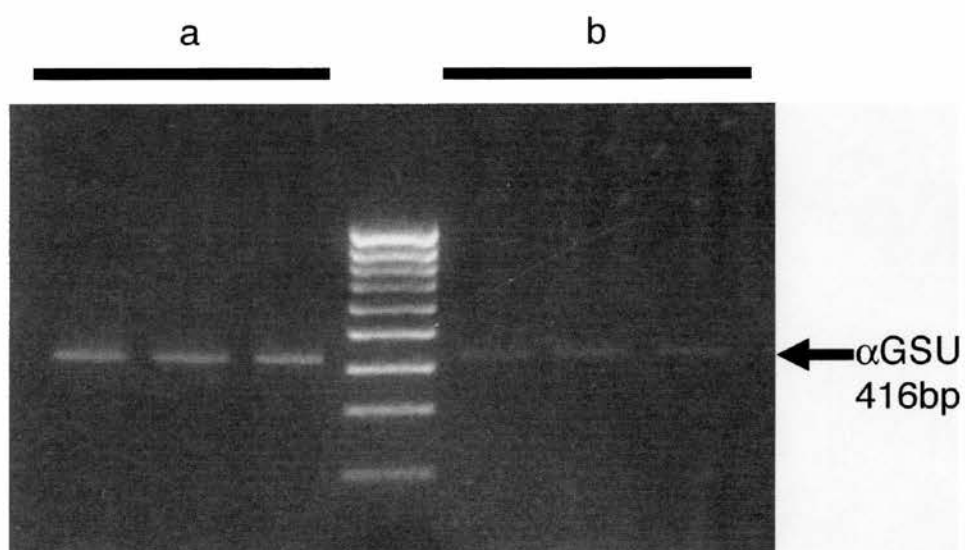


Fig. 2.5.3h[i]: PCR reamplification of α GSU with one or two units of AmpliTaq.

PCR was performed using sheep pituitary DNA, α GSU specific primers, and one (b) or two (a) units of AmpliTaq DNA Taq polymerase to amplify a 416bp fragment. Since two units of AmpliTaq increased the yield by two-fold, this enzyme was not working efficiently.

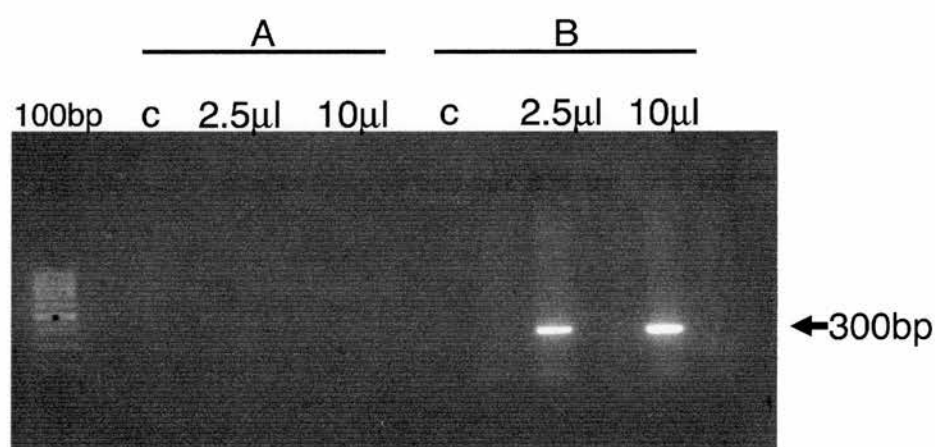


Fig. 2.5.3h[ii]: PCR reamplification of DD-RT-PCR isolated DNA fragments using AmpliTaq (A) or Pfu polymerase (B) and two rounds of 40 cycles of PCR.

DNA fragments were reamplified using unmodified differential display primers, and either AmpliTaq or Pfu polymerase. A control of one round of PCR was included (c). The volumes correspond to the volume of first round PCR product transferred to the second round of PCR.

tagged) were actually priming the DNA fragments, PCR experiments were set up with; the unmodified or the modified primers for both rounds of PCR, and the unmodified and modified primers for the first and second rounds of PCR respectively (Fig. 2.5.3h[iii]). In this experiment the modified primers did not appear to be annealing to the DNA fragments. Changing the PCR conditions (annealing temperatures, and MgCl_2 concentrations) failed to generate any product when the modified primers were used, therefore it was decided that the unmodified primers would be used for re-amplification, and the products would then be cloned. Initially AGS Gold™ was used to re-amplify the DNA isolated from DD-RT-PCR gels (Fig. 2.5.3h[iv]), and the protocol described below was optimised for PCR with this Taq polymerase.

The PCR components were prepared as a master mix on ice as follows;

4 μl 10x buffer
2.5 μl 25mM MgCl_2
4 μl Enhancer
2.5 μl 20 μM T_{12}VN
3.2 μl 10mM dNTP
0.3 μl AGS Gold
11 μl water

and 27.5 μl of the master mix was added with 2.5 μl of the relevant 5' primer to the DNA. The PCR was performed in a DNA Engine PTC-200 (GRI) under the following conditions; 94°C for 2 minutes, then 40 cycles of 94°C for 30 seconds, annealing at 40°C for 2 minutes, extension at 72°C for 30 seconds, with a final extension at 72°C for 5 minutes, and cooled at 4°C indefinitely. The PCR reaction was spun briefly and split into four 10 μl aliquots, which were re-amplified using the same reaction conditions as described above, this ensured that the PCR volume was kept to a minimum, and increased the likelihood of the PCR working during the second round of

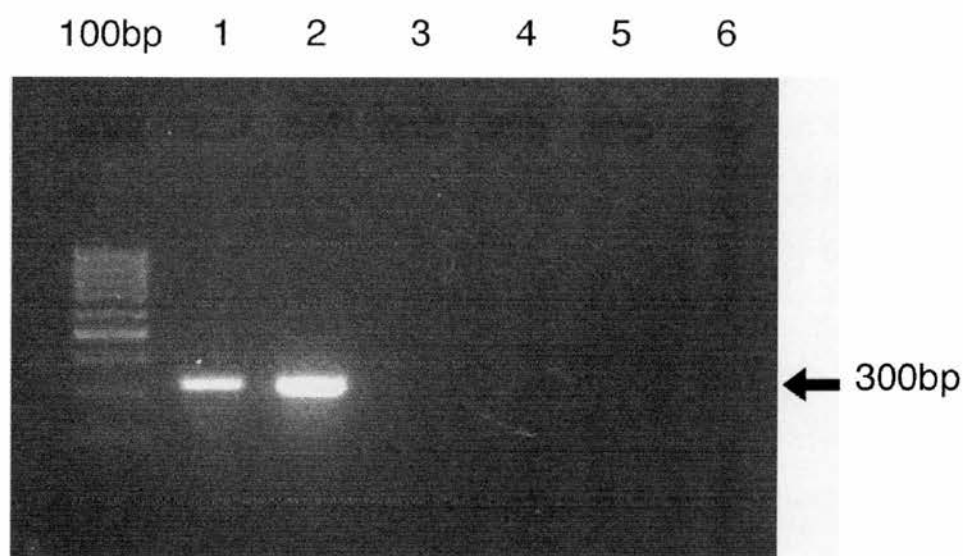


Fig. 2.5.3[iii]: Re-amplification of DD-RT-PCR isolated fragments using Pfu polymerase.

DD-RT-PCR products were re-amplified using; unmodified primers for both rounds of PCR (lanes 1 and 2), unmodified primers for the first round of PCR and restriction site tagged (modified) primers for the second round of PCR (lanes 3 and 4), and modified primers for both rounds of PCR (lanes 5 and 6). A 300bp PCR product could only be visualised with the unmodified primers.

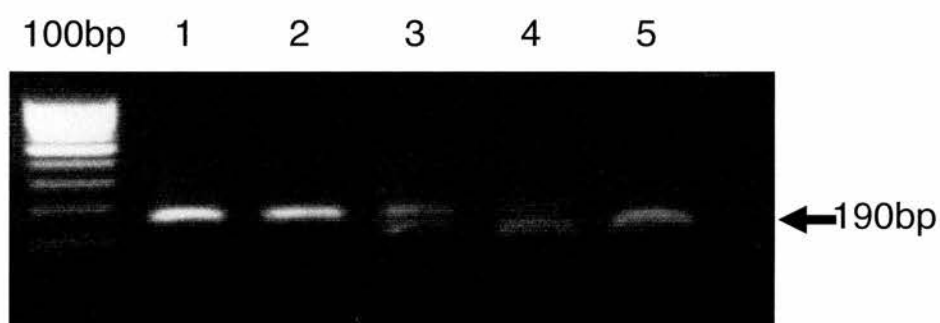


Fig. 2.5.3h[iv]: Reamplification of DD-RT-PCR fragments using AGS Gold Taq polymerase.

DD-RT-PCR products were amplified using AGS Gold Taq polymerase, R5 and T₁₂VC primers, and two rounds of 40 cycles of PCR. A 5µl aliquot was electrophoresed on this 2% agarose gel to visualise the PCR products. Lane 3 contains two bands, and was gel purified.

PCR. The reactions were pooled, and a small aliquot was run on an agarose gel (Fig. 2.5.3h[iv]). If multiple bands were present, the sample was gel purified using the Hybaid gel purification kit (Hybaid), and the PCR product of the expected size was eluted and cloned, otherwise the sample was cloned directly, using the Perfectly Blunt cloning kit (Novagen). The production of smaller PCR products, than would be predicted from the DD-RT-PCR gel, was a consistent feature of AGS Gold™ (Fig 2.5.3h[va]) and was considered a problem. So, the protocol was further modified to use a Taq DNA polymerase mix that included a combination of Taq DNA polymerase, Pfu DNA polymerase and dNTPs. The High fidelity extensor mix (AB Gene) improved both yield of PCR product and generated longer DNA fragments in comparison to AGS Gold™ (Fig. 2.5.3h[va]). The re-amplification was carried out using:

20μl 2x PCR mix
10μl DNA
2.5μl 20μM random primer
2.5μl 20μM T₁₂ VN
5μl water

initially for 40 cycles (Fig. 2.5.3h[va]), and although the PCR conditions remained the same, the number of amplification cycles had to be reduced to 12 due to the high processivity and efficiency (Fig. 2.5.3h[vb]). As before, this initial reaction was split into 4 and the PCR repeated.

2.5.3i Cloning and Identification of the PCR product

The products were cloned (Fig 2.5.1, Step 11-12) using the Perfectly Blunt Cloning kit, as described in the previous section (2.3.9b) and sequenced. DNA sequences were compared to the EMBL and rodent EST database using

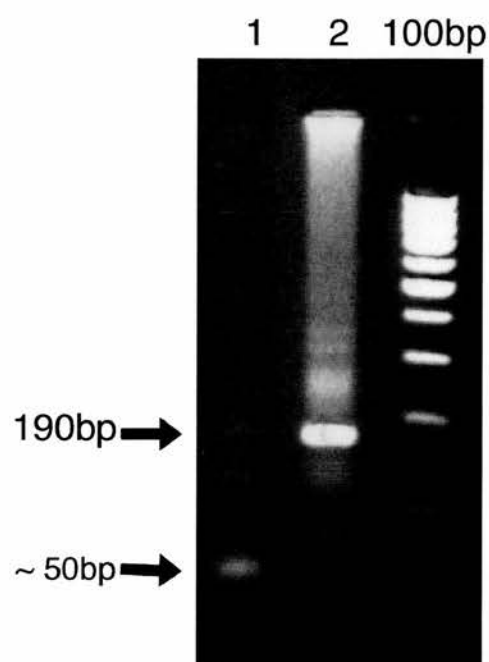


Fig. 2.5.3h[va]: A comparison of the processivity and efficiency of AGS Gold Taq polymerase and high fidelity extensor PCR mix.

A DD-RT-PCR gel was electrophoresed with identical differential display reactions in duplicate. The same band from each duplicate was chosen, the DNA isolated, and reamplified with two rounds of 40 cycles of PCR and either AGS Gold Taq polymerase (lane 1), or high fidelity extensor PCR mix (lane 2).

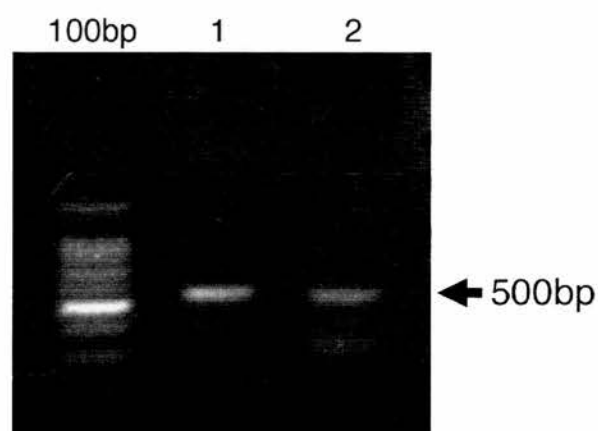


Fig. 2.5.3h[vb]: Reamplification of DD-RT-PCR fragments using the high fidelity extensor PCR mix.

DD-RT-PCR fragments were amplified with R1 and T₁₂VC and 2 rounds of 12 cycles of PCR. A 5 μ l aliquot was electrophoresed on this 2% agarose gel. The smaller number of rounds has removed the smear seen in the previous gel, and produced larger fragments.

a BLAST search (Wisconsin package version 10.0, Genetics Computer Group (GCG), Madison, Wisc).

2.5.3j Analysis and confirmation of differential mRNA expression patterns of clones

Transcripts were initially analysed by hybridisation of a radiolabelled probe, generated from the clones, to a Southern blot (see section 2.3.12 for protocol), to determine the suitability of the clone to be used as a probe for Northern analysis (for protocol see section 2.3.17), or RPA analysis (see section 2.3.18 for protocol, Fig. 2.5.1, Step 13).

Chapter 3:

Identification of GnRH regulated mRNA transcripts

3.1 Introduction

3.1.1 Background

The oestrus cycle is the recurring period of sexual receptivity and fertility in female mammals, and is characterised by cyclical changes in luteinising hormone (LH), follicle stimulating hormone (FSH), oestradiol and progesterone levels (see Fig. 1.1.2a). During each cycle, the female reproductive tract is prepared for the fertilisation and implantation of an ovum released from the ovary at ovulation and is divided into two phases, the follicular and luteal phase.

FSH and LH therefore, are differentially regulated during the oestrus cycle by the steroid hormones, but more importantly, by GnRH (Marshall, Dalkin et al. 1991). During the follicular phase, GnRH is secreted from the hypothalamus at a rate of one pulse every 15 minutes, and this rate dramatically increases towards the LH surge. In the luteal phase, the rate of GnRH secretion falls to about one pulse every hour (Knobil 1980; Lincoln, Fraser et al. 1985; Clarke, Thomas et al. 1987). Numerous studies have identified the importance of GnRH pulse frequency in differentially controlling the transcription and mRNA expression of the gonadotrophin subunits. Initial experiments were carried out *in vivo*, in ovariectomised and hypothalamic-pituitary disconnected sheep. These experiments identified that in order to maintain LH β and FSH β mRNA levels, the sheep had to

receive pulses of GnRH, since a constant administration of GnRH resulted in a 50% drop in mRNA levels (Mercer, Clements et al. 1988). These experiments were recapitulated in rats and mice, which identified that LH β mRNA expression levels were actually dependent on a specific pulse frequency, deviation from this frequency led to a decrease in LH β mRNA levels (Papavasiliou, Zmeili et al. 1986). Varying pulse frequencies of GnRH administered to the anterior pituitaries of ovariectomised or intact rats identified that LH β and α GSU mRNA levels were increased with rapid pulses of GnRH, while slow pulses up-regulated FSH β mRNA levels (Dalkin, Haisenleder et al. 1989; Shupnik 1990; Weiss, Duca et al. 1990; Haisenleder, Dalkin et al. 1991). Furthermore, there is a dissociation between LH β transcription and LH secretion indicating that GnRH pulse frequency differentially controls these events (Salton, Blum et al. 1988; Weiss, Duca et al. 1990; Weiss, Crowley et al. 1992).

Since GnRH pulse frequency is responsible for differential expression of the gonadotrophin subunits (Kaiser, Jakubowiak et al. 1997), then logically, the factors involved in controlling synthesis of LH β , FSH β and α GSU would also be regulated differently with different GnRH pulse frequencies. In fact, GnRH receptor numbers also increase with pulsatile administration of GnRH (Katt, Duncan et al. 1985). Additionally, GnRH-R numbers are affected by pulse frequency; there are fewer receptors expressed in response to a fast GnRH pulse frequency than a slow GnRH pulse frequency (Kaiser, Jakubowiak et al. 1997). However, receptor numbers have been observed to increase during the period leading up to the LH surge (Savoy-Moore, Schwartz et al. 1980; Barkan, Regiani et al. 1983; Brooks, Crow et al. 1992).

GnRH regulated transcription has also been studied *in vitro* using *in vivo* transformed cell-lines. Until recently, most experiments have been carried out using α T3-1 cells, which are an early gonadotroph cell-line that synthesise and secrete α GSU and express GnRH-R, but do not express the β -subunits (Windle, Weiner et al. 1990). L β T2 cells were developed in 1996, and are also of a gonadotroph lineage. These cells recapitulate differentiated gonadotrophs and can synthesise and secrete LH and FSH in response to GnRH (Turgeon, Kimura et al. 1996; Graham, Nusser et al. 1999). Pulsatile treatment of L β T2 cells with GnRH has been shown to induce expression of LH β mRNA and to promote secretion of LH storage granules (Turgeon, Kimura et al. 1996). Interestingly, neither α GSU nor GnRH receptor mRNA levels were altered in response to 15 minute pulses of GnRH in these cells. However FSH β gene expression is only activated by treatment with activin and GnRH, suggesting involvement of the SMAD signalling pathway in these cells (Graham, Nusser et al. 1999; Lebrun, Takabe et al. 1999; Pernasetti, Vasilyev et al. 2001). Therefore these cells mimicked *in vivo* expression of gonadotrophins in an *in vitro* model system. Although it is known that the GnRH receptor number is differentially regulated, the differences downstream of this are not known. There is a significant gap in the literature regarding the regulation of GnRH action. Although some second messenger signalling pathways have been implicated, there is a deficit of information on how this could differentially control and express two different hormones from the same cell and how all of this is controlled by pulsatile input of GnRH.

3.1.2 The Aims of this Chapter

Thus, the *in vivo* transformed gonadotroph cell line L β T2 was chosen to investigate changes in gene expression induced by GnRH, with the specific aim of discovering new and novel factors regulated by GnRH, that would also explain how GnRH can differentially modulate biosynthesis and secretion. L β T2 cells are GnRH responsive, express LH β , FSH β and α GSU (Turgeon, Kimura et al. 1996; Graham, Nusser et al. 1999) and are naïve to GnRH, therefore *de novo* synthesis in response to GnRH could also be studied. Using a cell-line ensured that GnRH administration was controlled and consequently allowed the effect of different pulse regimes to be studied. To try and identify transcripts that are regulated by GnRH, L β T2 cells were either left untreated or treated with GnRH. RNA was isolated from these cells and reverse transcribed before being used for DD-RT-PCR analysis (Liang and Pardee 1992).

3.2 Results

3.2.1 GnRH but not Matrigel™ has an effect on transcription

L β T2 gonadotroph cells are grown *in vitro* on a Matrigel™ basement membrane mix. Matrigel™, as well as containing collagen and other proteins found in extracellular basement membranes, contains a number of growth factors, metalloproteinases and other factors that promote cell adherence. These factors may up-regulate gene expression. Therefore differential display was performed to identify if gene expression was affected by Matrigel™ in L β T2 cells. The cells were also treated with GnRH to confirm that mRNA expression altered in response to GnRH (Fig. 3.2.1).

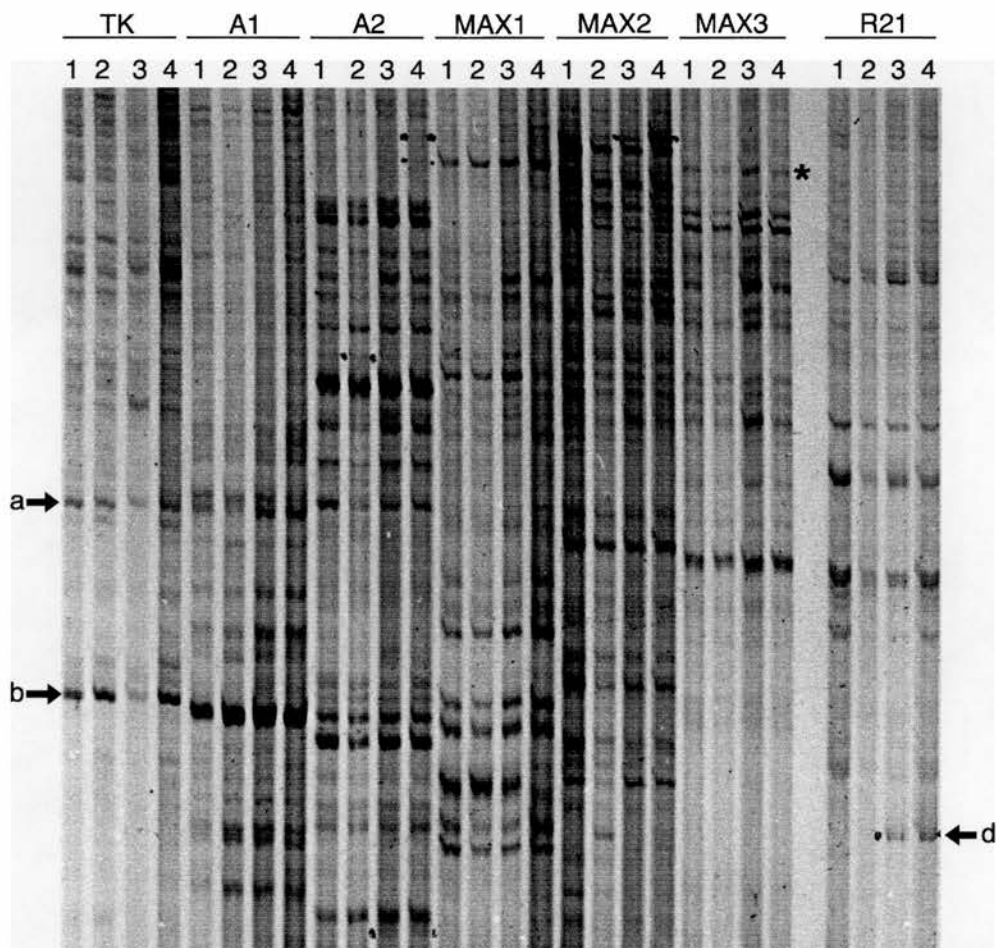


Fig. 3.2.1: Differential display showing that GnRH, not matrigel, has an effect on gene expression.

LβT2 cells were treated as follows: no GnRH or matrigel (lane 1); no GnRH, with matrigel (lane 2); with 3 pulses of GnRH and with matrigel (lane 3); with 6 pulses of GnRH and with matrigel (lane 4). The differential display RT-PCR was performed using the specified primers and T12VA anchored primers. Arrows show transcripts that are not affected by matrigel (a and b), and others that are up-regulated by GnRH (* and d).

Cells were left untreated, and left without Matrigel™, (Lanes 2 and 1 respectively), or seeded on Matrigel™ (section 2.4.3) with three 15 minute pulses of GnRH over one day (section 2.4.8), or six 15 minute pulses of GnRH over two days (Lane 3 and 4 respectively). The differential display gel showed that the Matrigel™ had no effect on gene expression, whereas GnRH up-regulated gene expression.

3.2.2 Myosin Light Chain mRNA is regulated by GnRH

Having established that GnRH up-regulated mRNA expression levels, further experiments were carried out to investigate this. Cells were either left untreated (lane 1) or treated with GnRH for either one 15 minute pulse (lane 2), or two 15 minute pulses of GnRH with an inter-pulse interval of either 30 minutes (lane 3, follicular phase) or 90 minutes (lane 4, luteal phase). Cells were harvested 4 hours later, the RNA extracted (section 2.3.14) and DD-RT-PCR (section 2.5.3a) performed (Fig 3.2.2a). The band illustrated appeared to be up-regulated after one pulse of GnRH, down-regulated after two pulses of GnRH 30 minutes apart, and again up-regulated after 2 pulses of GnRH 90 minutes apart. This band was isolated (section 2.5.3f), reamplified using AGS Gold™ Taq polymerase (section 2.5.3h), cloned into psTBlue-1 (section 2.3.9b) and sequenced (section 2.3.11c). The 212bp sequence was compared to the EMBL database using BLAST (section 2.5.3i) and a match was found with myosin light chain (Fig. 3.2.2b). A probe was generated from the clone and Northern analysis on 10µg total RNA (section 2.3.17), from the same RNA samples used to generate the cDNAs used in DD-RT-PCR, was performed (Fig. 3.2.2ci). Initially, the pattern of mRNA expression

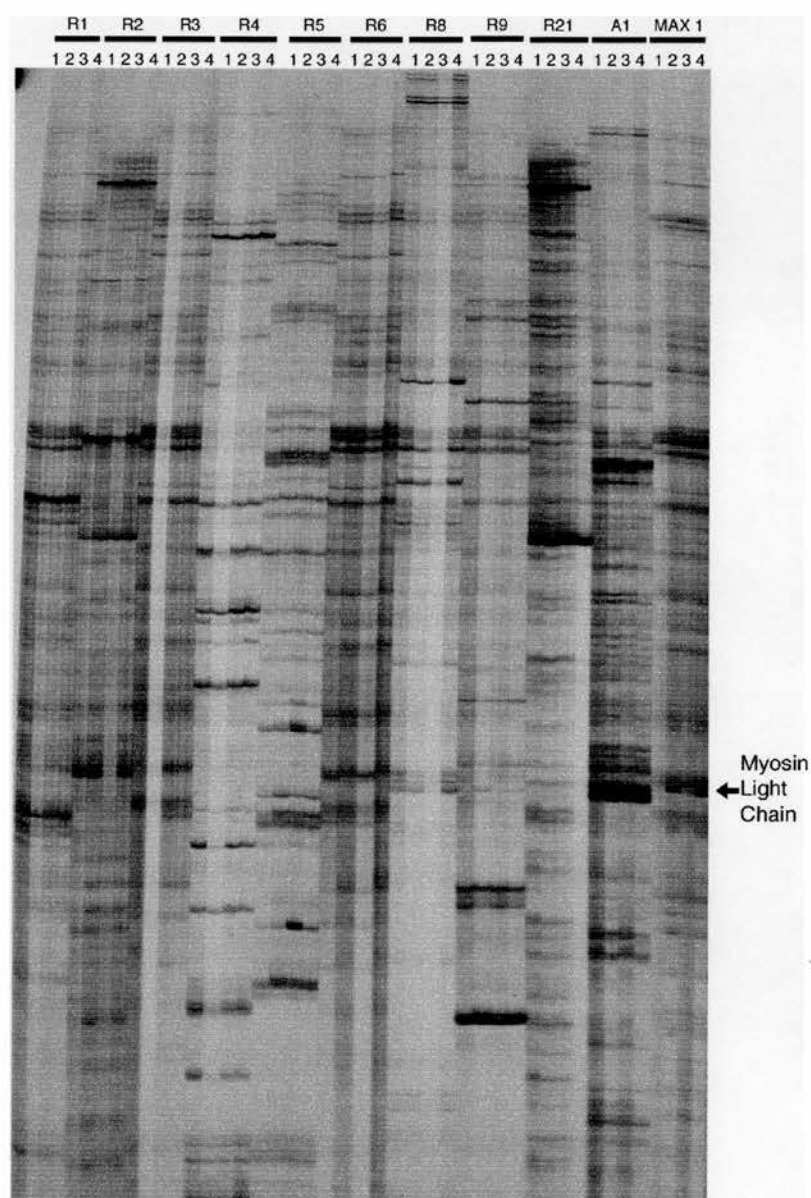


Fig. 3.2.2a: Differential display gel showing changes in expression profiles of cells either left untreated, or treated with GnRH.

LβT2 cells were left untreated (lane 1), or treated with 1 pulse of GnRH (lane 2); or 2 pulses of GnRH 30 minutes apart (lane 3); or 2 pulses of GnRH 90 minutes apart (lane 4). Differential display was performed using the primers specified and T12VC anchored primers. The arrow identifies Myosin light chain which was up-regulated in response to 1 pulse, and 2 pulses of GnRH 90 minutes apart.

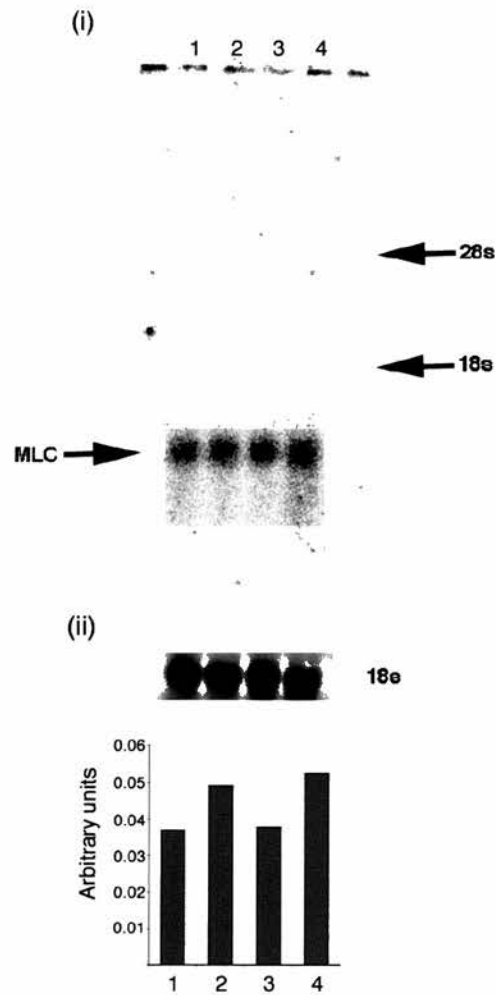


Fig. 3.2.2c: Northern analysis of myosin light chain to confirm the mRNA expression profile identified by differential display.

- (i) RNA was extracted from LβT2 cells that were treated as follows: no GnRH (lane 1); 1 pulse of GnRH (lane 2); 2 pulses of GnRH 30 minutes apart (lane 3); and 2 pulses of GnRH 90 minutes apart (lane 4) using RNazol B. A 10μg aliquot of each sample was loaded per lane. The membrane was hybridised with a radiolabelled probe generated from the cloned differential display product.
- (ii) The same northern blot was stripped, and reprobed using an 18s probe. This was used to quantify the hybridised bands produced by the myosin light chain.

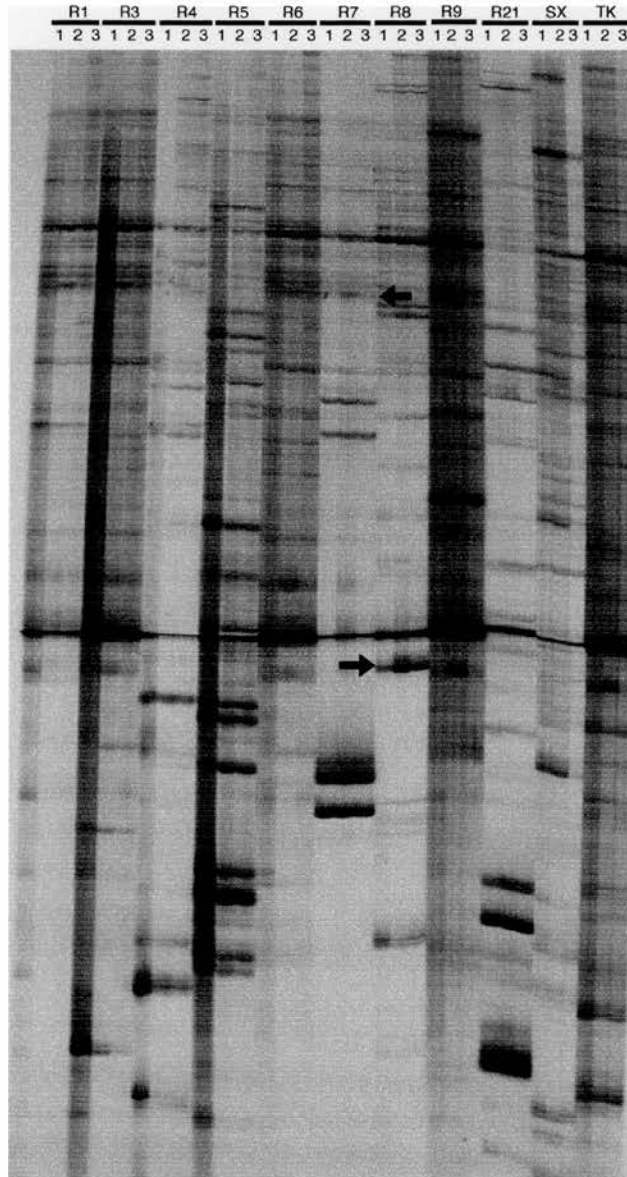


Fig. 3.2.2d: Differential display gel showing mRNA expression patterns 4 and 6 hours post-GnRH treatment.

LβT2 cells were treated as follows: no GnRH (lane 1); 1 pulse of GnRH; harvesting the RNA 4 hours later (lane 2); and 1 pulse of GnRH, harvesting the RNA 6 hours later (lane 3). Differential display RT-PCR was performed with the specified primers and T12VA anchored primer. The arrows mark the typical mRNA expression profiles obtained using this regime.

appeared unchanged, however quantification with an 18S ribosomal control probe confirmed that the mRNA profile matched the differential display pattern, but the profile was not as defined as that originally seen on the DD-RT-PCR gel (Fig. 3.2.2cii). Although differences were identified using this pulse regime, they were difficult to interpret since the expression patterns appeared on the differential display gel as on/off signals, but further analysis by northern blotting showed this not to be the case. With this in mind, a simpler regime was adopted, involving a single GnRH pulse of 15 minutes. Initially, the RNA was harvested 0, 4 and 6 hours post-GnRH treatment, however the mRNA expression profiles showed that transcripts were induced by 4 hours, but had waned by 6 hours (Fig. 3.2.2d).

3.2.3 GnRH regulation of gene expression is rapid

Therefore, a shorter time course was used, and RNA was harvested 0, 1, 2 and 4 hours post-GnRH treatment. DD-RT-PCR was performed and numerous differences in transcript levels were identified. The time course experiment and DD-RT-PCR were carried out in duplicate. Samples were loaded on the same gel, to enable expression profiles to be compared and only differences that were consistent between the duplicates were isolated and analysed. The results described below showed that mRNA expression levels changed rapidly, within one hour of GnRH treatment and that in general, the mRNA expression levels were up-regulated in response to GnRH.

3.2.4 DD-RT-PCR produced fragments that contained nucleotide repeat sequences.

Having identified a GnRH pulse regime that generated reproducible results, the first transcripts isolated tended to contain nucleotide repeats, which are common in the 3' untranslated region sequences (3' UTR) of mRNA. These clones when used as probes for northern blots, hybridised to numerous transcripts, which presumably contained repeat sequences, this produced a smear on the blot that was impossible to interpret (Fig 3.2.4a). Thus, it was decided that clones would be screened by both Southern and bioinformatic analysis, before continuing with further expression analysis. Using this screen, one clone was identified as unsuitable for further analysis by northern blot, or ribonuclease protection assay (RPA, Fig 3.2.4b), however five other clones that passed this screening test were investigated and are described below.

3.2.5 DD-RT-PCR has identified a novel sequence that is regulated by GnRH

Differential display PCR was performed using the R1 and T₁₂VC primer combination, and two changing bands were identified with similar expression patterns, these were named R13c and R18b (Fig. 3.2.5a, the results of R18b will be covered in section 3.2.6). The expression profile suggested a rapid up-regulation of expression within 1 hour, which peaked within 2 hours, returning to pre-GnRH treatment levels by 4 hours. The DNA was isolated, amplified with the High Fidelity Extensor Taq polymerase (section 2.5.3h), cloned into psTBlue-1 (section 2.3.9b), and sequenced (section 2.3.11b). R13c (279bp) was compared with the high throughput sequencing database using a BLAST search (section 2.5.3i), and an exact match was

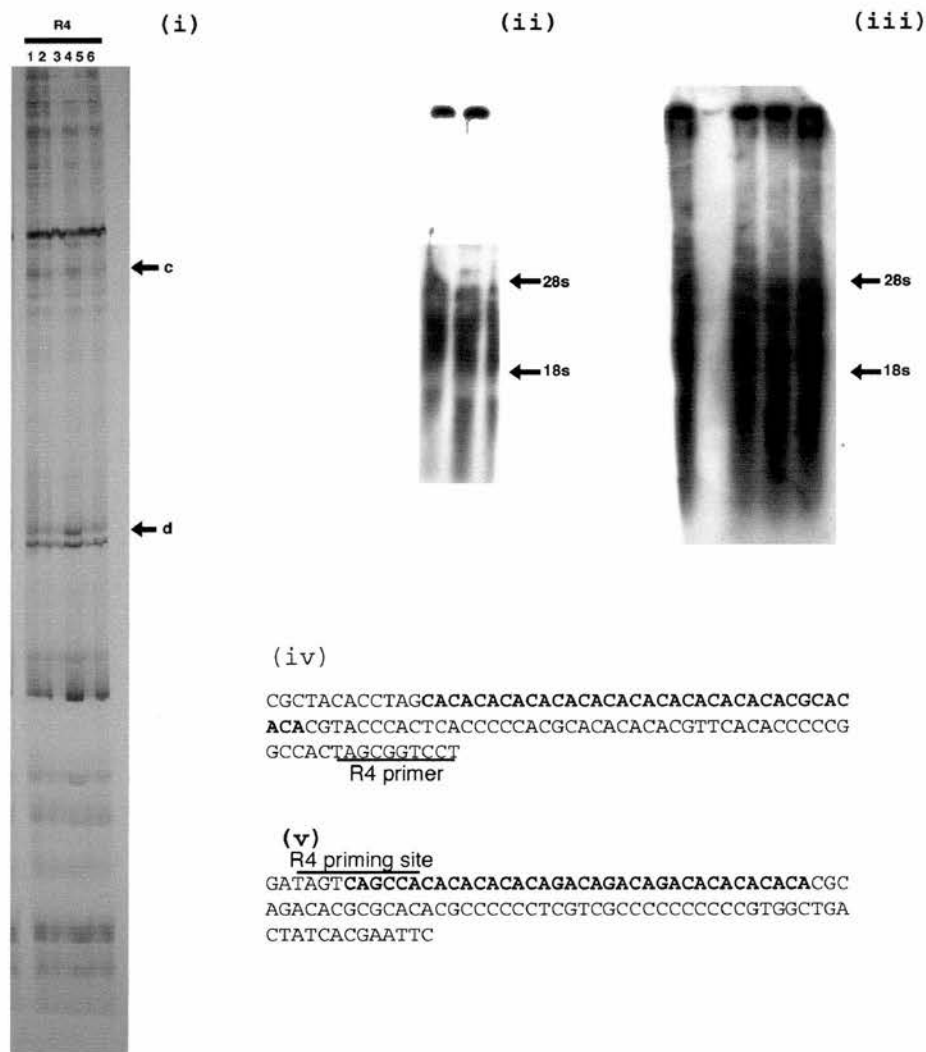


Fig. 3.2.4a: Investigation of cloned differential display products identified as containing repeat DNA sequences as probes for Northern analysis.

- (i) Differential display RT-PCR was performed on RNA that had been isolated from cells treated as follows: no GnRH (lane 1); 1 pulse of GnRH harvesting 4 and 6 hours later (lane 2 and 3); 2 pulses of GnRH 30 minutes apart harvesting 4 and 6 hours later (lane 4 and 5); and 2 pulses of GnRH 90 minutes apart harvesting 4 hours later (lane 6). A R4, T12VA primer combination was used.
- (ii) Northern hybridisation of a radiolabelled probe generated from clone d
- (iii) Northern hybridisation of a radiolabelled probe generated from clone c
- (iv) Full length DNA sequence of clone d, the repeat sequence has been highlighted in bold and the primer sequence underlined.
- (v) Full length DNA sequence of clone c, the repeat sequence has also been highlighted in bold, and a sequence similar to the R4 primer has been identified.

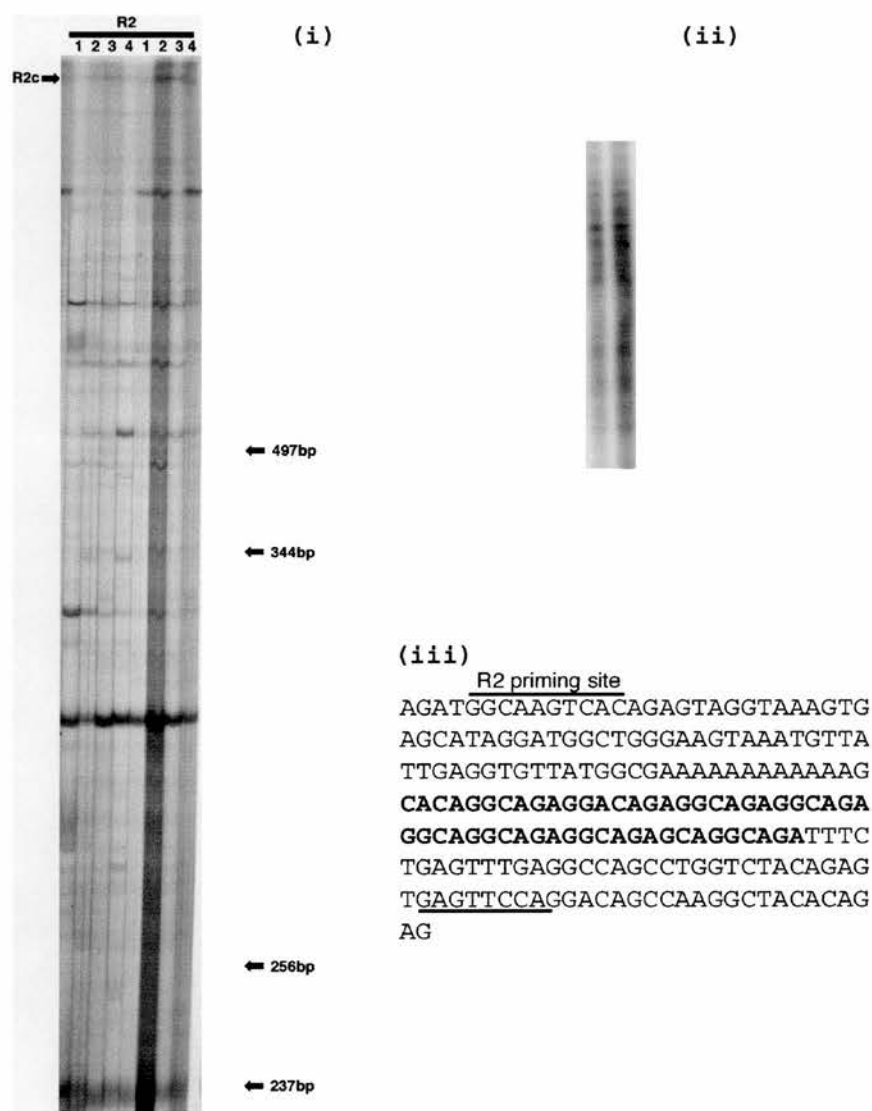


Fig. 3.2.4b: The R2c clone detects multiple discrete bands when used to probe a Southern blot.

- (i) Differential display gel identifying the R2c transcript. L β T2 cells were treated as follows: no GnRH (lane 1); or 1 pulse of GnRH harvesting 1 hour (lane 2); 2 hours (lane 3); and 4 hours (lane 4) post-GnRH treatment. Differential display was performed using the R2 and T12VC primers.
- (ii) Southern blot analysis of *Bam*HI and *Hind*III restricted mouse genomic DNA revealed that the R2c probe generated from the cloned differential display product produced multiple hybridising bands.
- (iii) The 205bp sequence reveals a 54bp stretch of repeat sequence (highlighted in bold) and the R2 primer sequence is underlined. A region of DNA sequence has also been underlined and denotes a possible PCR mispriming site.

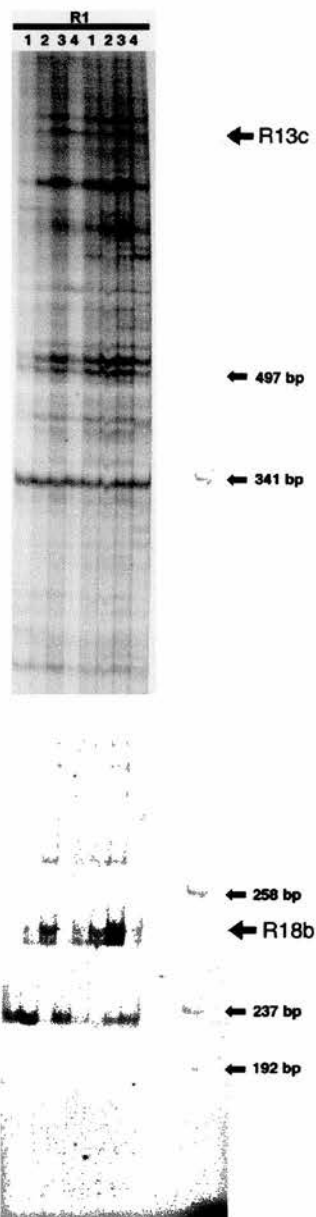


Fig. 3.2.5a: Differential display gel showing transcripts R13c and R18b are up-regulated in response to GnRH.

L β T2 cells were treated as follows: no GnRH (lane 1); or 1 pulse of GnRH harvesting 1 hour later (lane 2); 2 hours later (lane 3); and 4 hours later (lane 4). DD-RT-PCR was performed using the R1 and T12VC primers. Arrows denote radiolabelled DNA size markers and transcripts R18b and R13c.

Fig. 3.2.5b: DNA Sequence line-up of R13c and a region on human chromosome 3.

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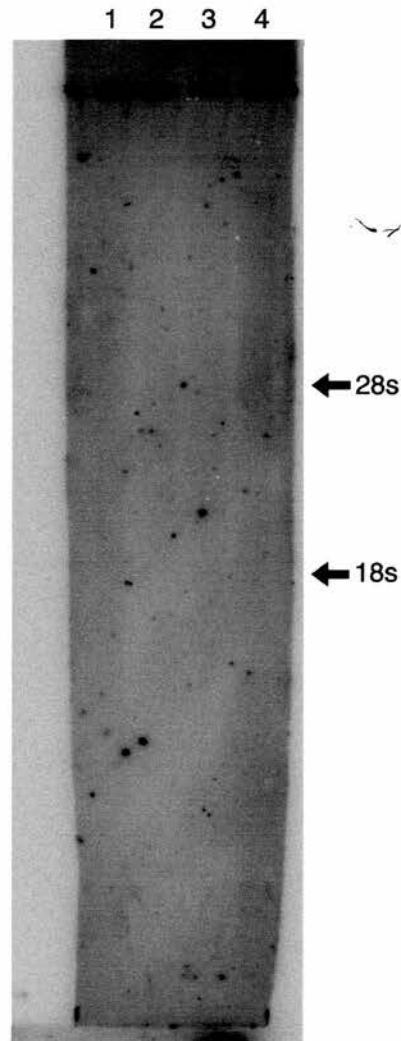


Fig. 3.2.5c: Northern blot analysis of R13c probe, no hybridising band was seen.

LβT2 cells were treated as follows: no GnRH (lane 1); or one pulse of GnRH harvesting at 1 hour (lane 2); 2 hours (lane 3); and 4 hours (lane 4). Total RNA was extracted from these cells, 10μg was loaded into each well, and electrophoresed on a 1% denaturing agarose gel. The RNA was transferred to a nylon membrane and hybridised with a radiolabelled DNA probe derived from the cloned differential display product R13c. The position of the ribosomal bands 28s and 18s are marked.

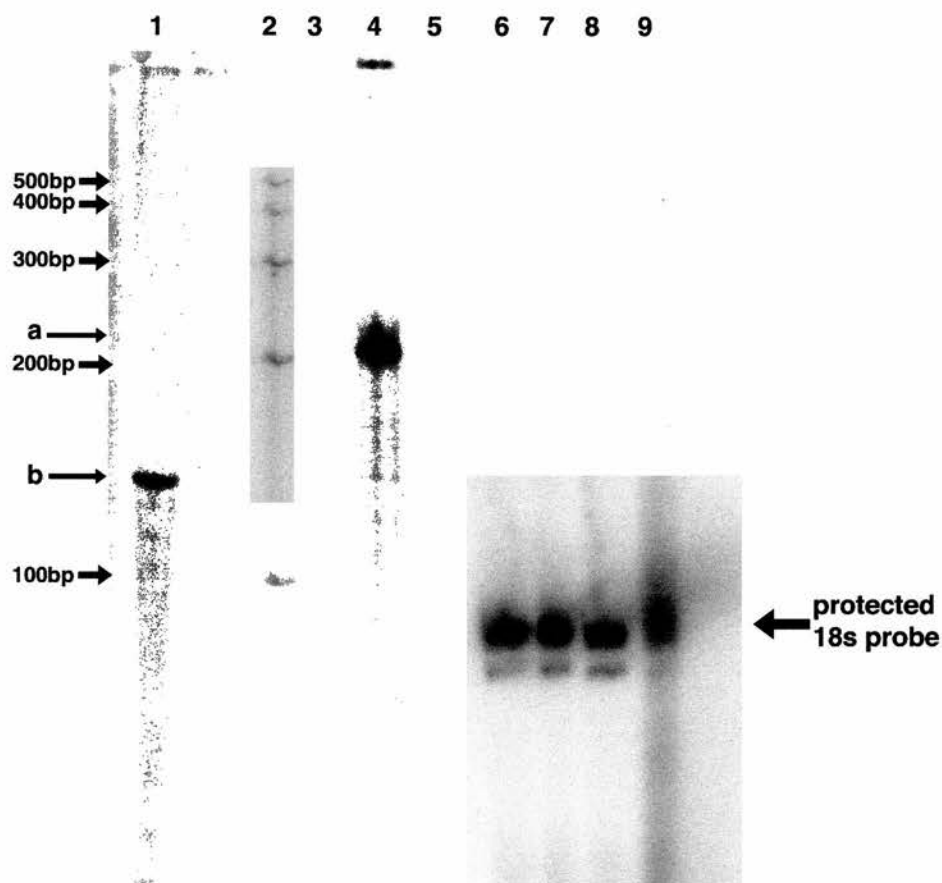


Fig. 3.2.5d: RPA analysis of R13c did not detect a hybridising band.

An RPA using a radiolabelled *in vitro* T7 RNA polymerase transcribed RNA probe of R13c was performed to confirm the mRNA expression profile of the differential display gel. LβT2 cells were treated as follows: no GnRH (lane 6); or 1 pulse of GnRH, harvesting at 1 hour (lane 7); 2 hours (lane 8); and 4 hours (lane 9) post-GnRH treatment. Arrows denote the unhybridised 18s probe (b, lane 1), the unhybridised R13c probe (a, lane 4). The 6% polyacrylamide gel was electrophoresed with a 100bp DNA size marker (lane 2) and unhybridised 18s and R13c probes, which have been treated with ribonuclease (lanes 3 and 5).

identified with a region on human chromosome 3 (Fig 3.2.5b). Northern blot analysis did not produce any hybridising bands on 10µg of total RNA (Fig 3.2.5c), therefore an RPA (section 2.3.18) was performed in an effort to confirm the differential display gel pattern however, RPA analysis did not produce any hybridising bands either (Fig. 3.2.5d).

3.2.6 DD-RT-PCR has identified a GnRH regulated transcript that is found in the adult male testis

R18b (132bp) was isolated from the same gel as R13c (section 2.5.3f), and had the same expression profile as R13c (Fig. 3.2.5a). Bioinformatics, however, identified this transcript as having similarity with an expressed sequence tag (EST), which is also present in the adult male testis (Fig. 3.2.6).

3.2.7 GnRH regulates a mRNA transcript that is found in the mammary gland, CNS and brain

This transcript (R3c, 503bp in length) was isolated using the R3 and T₁₂VC primers (section 2.5.3a). The mRNA expression profile suggests de novo synthesis by 1 hour post-GnRH treatment, which peaked by 2 hours post-GnRH treatment, and was sustained after 4 hours post-GnRH treatment (Fig. 3.2.7a). Bioinformatics has matched this clone with an EST in the Riken mouse database, which is expressed in the lactating and pregnant mouse mammary gland (Fig. 3.2.7b), and has some similarity with an EST that is only expressed in the hypothalamus, cerebellum, spinal cord, retina, diencephalon, medulla oblongata, hippocampus, and corpora quadrigemina.

R1 primer	
R1:b	<u>CCTTGAGGCA</u> CTCGTATTACAGTTTCCACATAAAATTACAAACAGGCAGGGGCAAAAAGTTTCTTCTATCAGTTTCTTCTATTTGATATTAG
Mouse	
Testis EST	GAGGCACTCGTATTACAGTTTCCACATAAAATTACAAACAGGCAGGGGCAAAAAGCCGCTCTGCATCGACATGAGAAAGTGATATTAG
R1:b	ATTTATTTTGAAGGAGGAACGTACCC <u>GC</u> TTTTTTTTTTTTTTTT
Mouse	
Testis EST	ATTTATTTTGAAGGAGGAACGTACC

Fig. 3.2.6a: DNA Sequence line-up of R18b against an EST found in the adult mouse testis.

R18b was cloned into pst-Blue1 and sequenced with T7 and SP6. A match was identified with an EST found in the adult mouse testis. The R1 and anchored primers are highlighted in bold and are underlined.

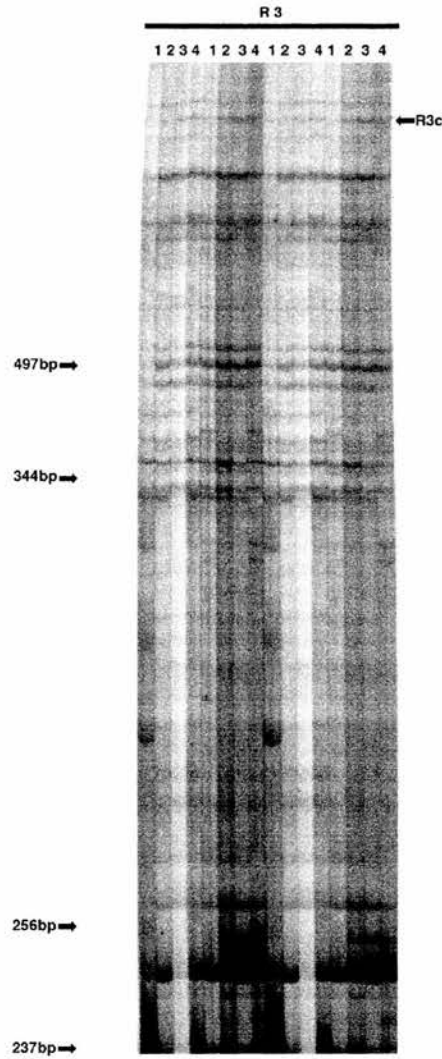


Fig 3.2.7a: Differential display gel identifying transcript R3c being up-regulated by GnRH.

L β T2 cells were treated as follows: no GnRH (lane 1); or 1 pulse of GnRH and harvested 1 hour (lane 2); 2hours (lane 3); and 4 hours (lane 4) post-GnRH treatment. Differential display was performed using the R3 and T12VC primer combination. Arrows denote the radiolabelled DNA size markers, and the R3c transcript.

TCGTCCTAATGTTGTAATAATCACTCAAAAAAGAAATAAAAGTCACCTGGTCTCTTGAAGTTTAA'CTTCTTAATAAAA'CATCTGTATGA4AAAA

TTAACTGAATGAACCTGTAAACCTCTCAGACTTGGGGAGGGGAAATTTATTACCTTAGTGATGTAAGTCCAAATTTGAA'AT'TAGT

T_{LV}C primer
ACCTGGGCTCTATCCTATTGATTTAGGTATTGACGCTTTTCTTTTCTTTT
ACCTGGTCTCTATCCTATTGATTTAGGTATTGACG

Fig. 3.2.7b: DNA Sequence line-up of R3c and a cDNA of a transcript found in ten day lactating and two day pregnant mammary gland

RR3c was cloned into *pst*-Blue1 and DNA sequenced using T7 and SP6 primers. A match was identified with a cDNA isolated from the mammary gland of a ten day lactating and a two day pregnant mouse. The anchored primer, and the possible R3 priming site are highlighted in bold and are underlined.

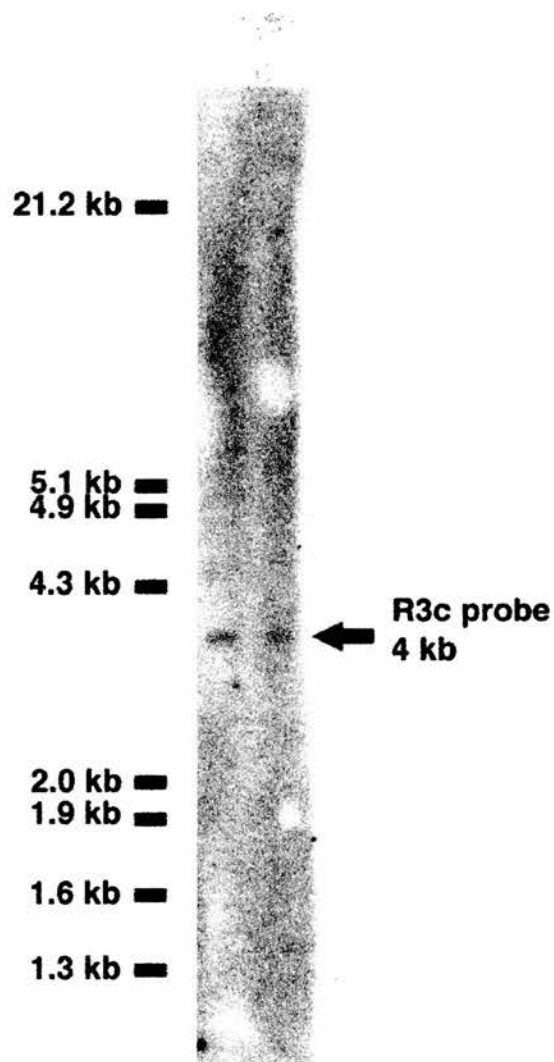


Fig. 3.2.7c: The R3c radiolabelled probe generated a discrete 4kb hybridising band on a Southern blot.

Mouse genomic DNA was digested with *Bam*HI and *Hind*III, and electrophoresed on a 0.8% agarose gel, and transferred to a nylon membrane. The membrane was probed with a radiolabelled probe generated from the cloned differential display product. The DNA marker corresponds to bacteriophage lambda DNA, restricted with *Hind*III and *Eco*RI

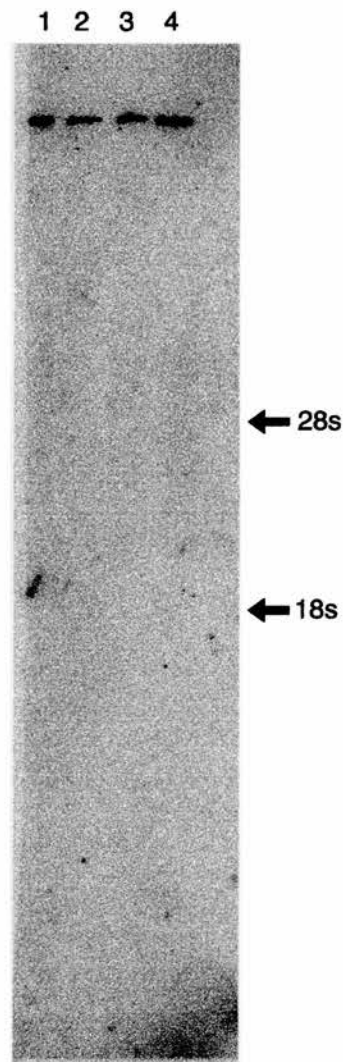


Fig. 3.2.7d: Northern blot analysis of R3c did not identify any hybridising bands.

LβT2 cells were treated as follows: no GnRH (lane 1); or one pulse of GnRH harvesting at 1 hour (lane 2); 2 hours (lane 3); and 4 hours (lane 4). Total RNA was extracted from these cells, 10μg was loaded into each well, and electrophoresed on a 1% denaturing agarose gel. The RNA was transferred to a nylon membrane and hybridised with a radiolabelled DNA probe derived from the cloned differential display product R3c. The position of the ribosomal bands 28s and 18s are marked.

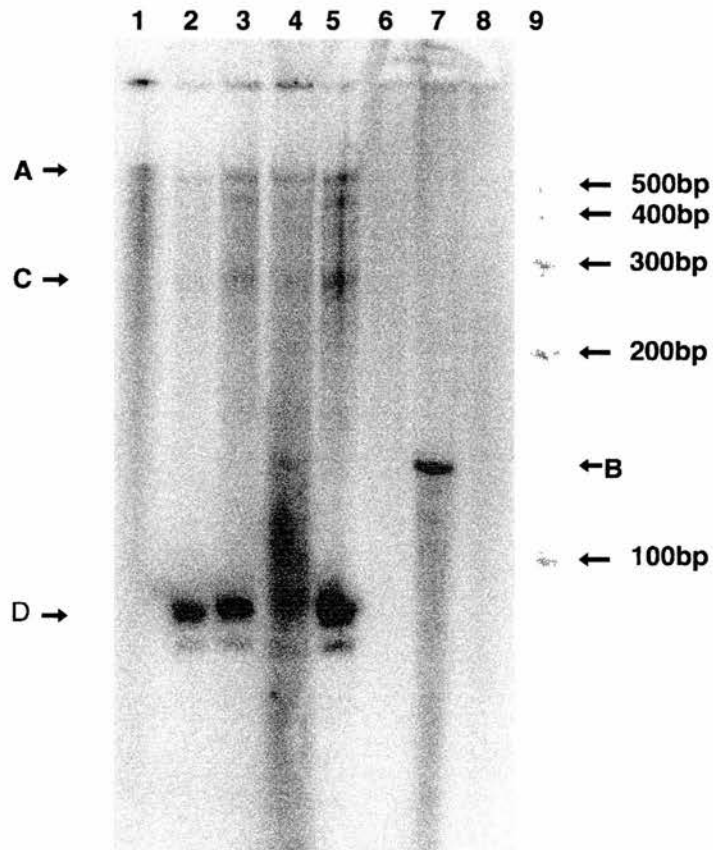


Fig 3.2.7e: RPA analysis of R3c transcript.

LβT2 cells were treated as follows: no GnRH (lane 2); or one pulse of GnRH harvesting at one hour (lane 3); 2 hours (lane 4); and 4 hours (lane 5) post-GnRH treatment. The cell lysate was hybridised with radiolabelled R3c and 18s *in vitro* translated RNA, and electrophoresed on a 6% polyacrylamide gel with unhybridised R3c and 18s probes (lanes 1 and 7, and arrows A and B respectively), and unhybridised R3c and 18s probe which have been treated with ribonuclease (lanes 6 and 8 respectively). Arrows C and D correspond to protected R3c and 18s bands respectively.

Southern blot analysis (section 2.3.12) identified a hybridising band of about 4.2kb (Fig. 3.2.7c), and confirmed that this clone was suitable as a probe for northern analysis, however, hybridisation of this probe to a northern blot with 10µg of total RNA, did not identify any bands (Fig 3.2.7d). Therefore an RPA was performed, but the results were inconclusive (Fig 3.2.7e).

3.2.8 DD-RT-PCR has identified a putative tyrosine phosphatase that is tightly regulated by GnRH

This clone, initially identified as R21c (133bp in length), was isolated from a differential display gel (section 2.5.3a) of reactions carried out with R21 and T₁₂VC primers (Fig 3.2.8a). The gel showed that expression was hugely up-regulated within one hour post-GnRH treatment, was maintained after two hours, and returned to pre-GnRH treatment levels by four hours. Bioinformatics identified a match at the 3' end of a putative tyrosine phosphatase (Fig. 3.2.8b). The Southern blot identified a single hybridising band of about 4.8kb, confirming the clone as a suitable probe for further analysis (Fig. 3.2.8c). RPA analysis confirmed the expression profile shown on the differential display gel (Fig. 3.2.8d) and quantification with 18s showed that this putative tyrosine phosphatase was up-regulated 27-fold in response to GnRH, with the mRNA expression levels waning after 4 hours (Fig. 3.2.8e).

3.2.9 DD-RT-PCR has identified that Fanconi's Anaemia Complementation Group A (FAA) is regulated by GnRH

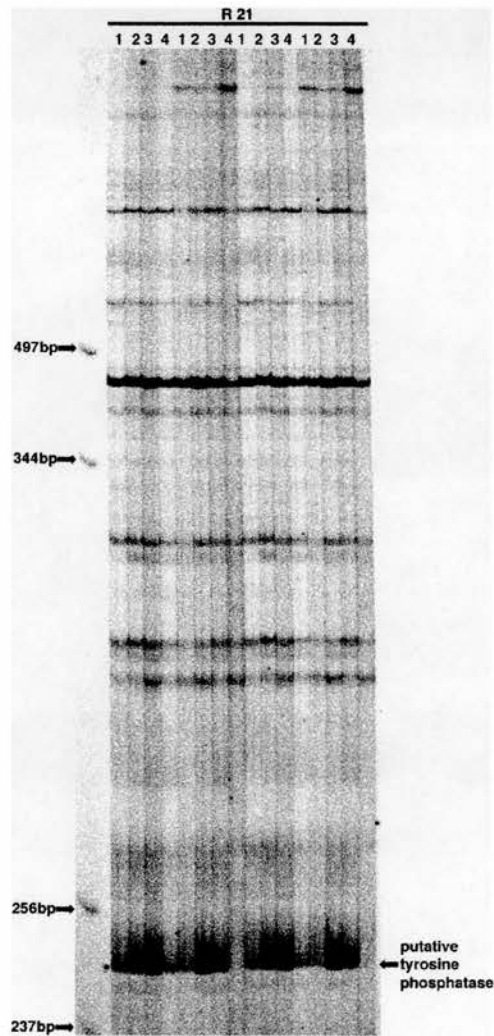
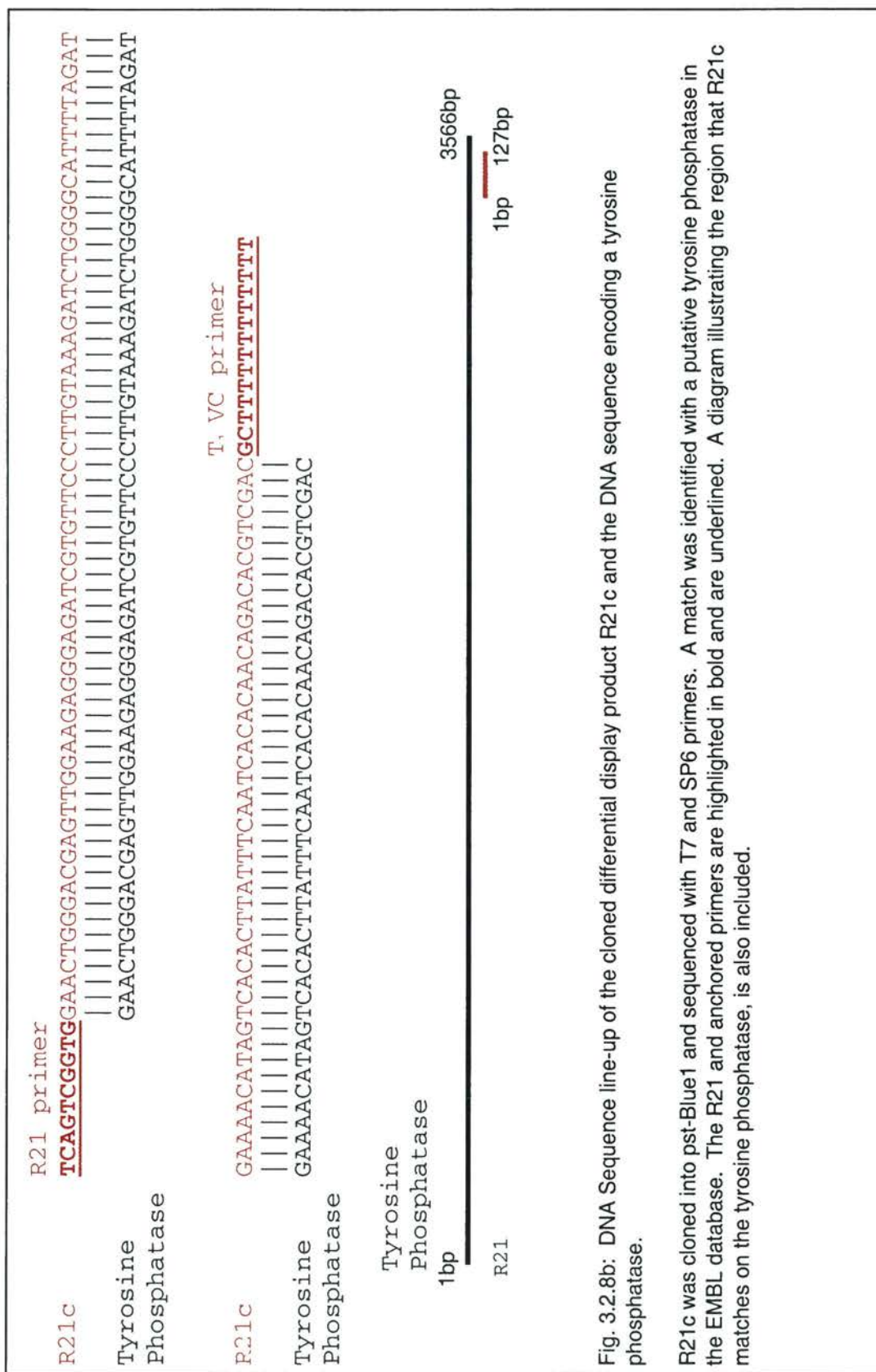


Fig. 3.2.8a: Differential display expression profile of a putative tyrosine phosphatase.

LβT2 cells were treated as follows: no GnRH (lane 1); or 1 pulse of GnRH and harvested 1 hour (lane 2); 2 hours (lane 3); and 4 hours (lane 4) post-GnRH treatment. Differential display was performed using the R21 and T12VC primer combination. The arrows denote the radiolabelled DNA size markers, and the putative tyrosine phosphatase profile.



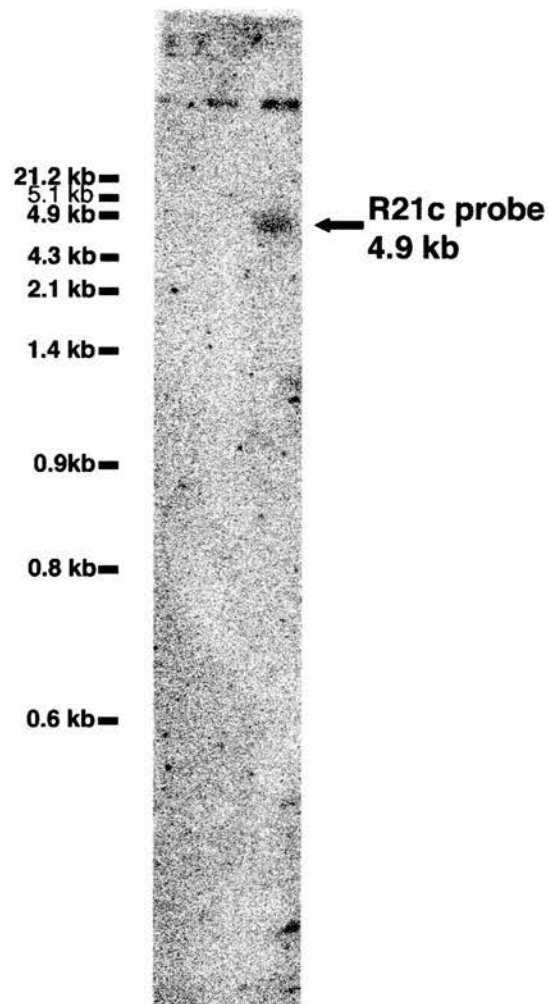


Fig 3.2.8c: The R21c probe from the cloned differential display product, generated a 4.9kb hybridising band on a Southern blot.

Mouse genomic DNA was digested with *Bam*HI and *Hind*III overnight, and electrophoresed on a 0.8% agarose gel, and transferred to a nylon membrane. The membrane was hybridised using a radiolabelled probe generated from the cloned differential display PCR product R21c, which has identity with a putative tyrosine phosphatase. The DNA size markers correspond to bacteriophage lambda restricted with *Eco*RI and *Hind*III.

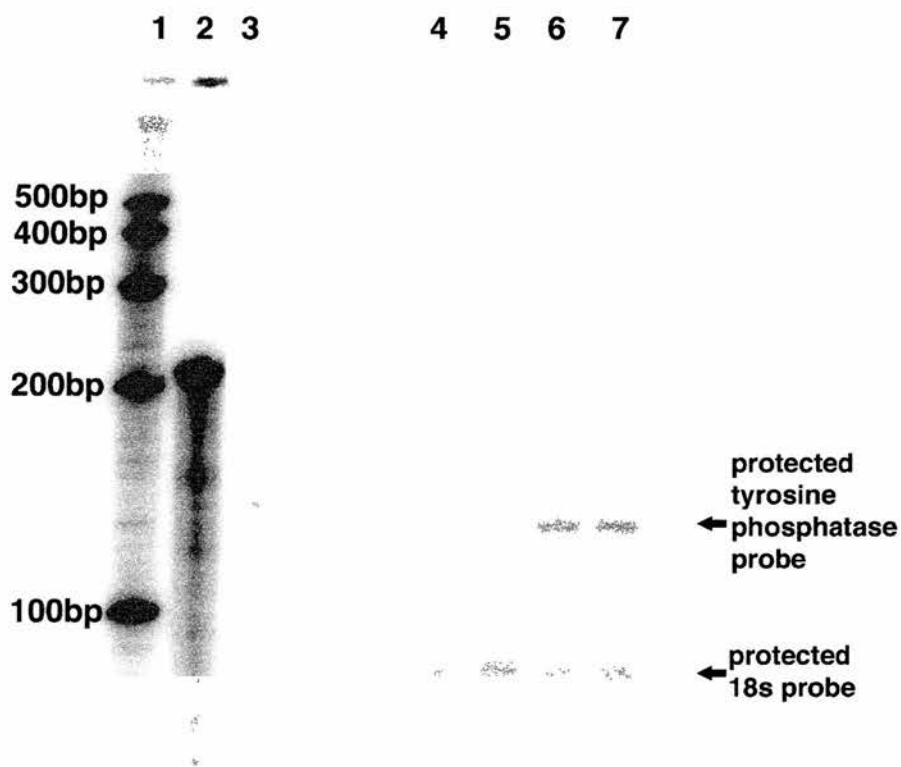


Fig. 3.2.8d: Confirmation of the mRNA expression profile by RPA analysis.

LβT2 cells were treated with; no GnRH (lane 4), or 1 pulse of GnRH, harvesting the cells into Direct Protect 1 hour (lane 5), 2 hours (lane 6) and 4 hours (lane 7) post GnRH treatment. An aliquot of the cell lysate was incubated with the radiolabelled tyrosine phosphatase and 18s RNA probes and electrophoresed on a 6% polyacrylamide gel with 100bp marker (lane 1), and the unhybridised tyrosine phosphatase probe (lane 2 and 18s probe (lane 3).

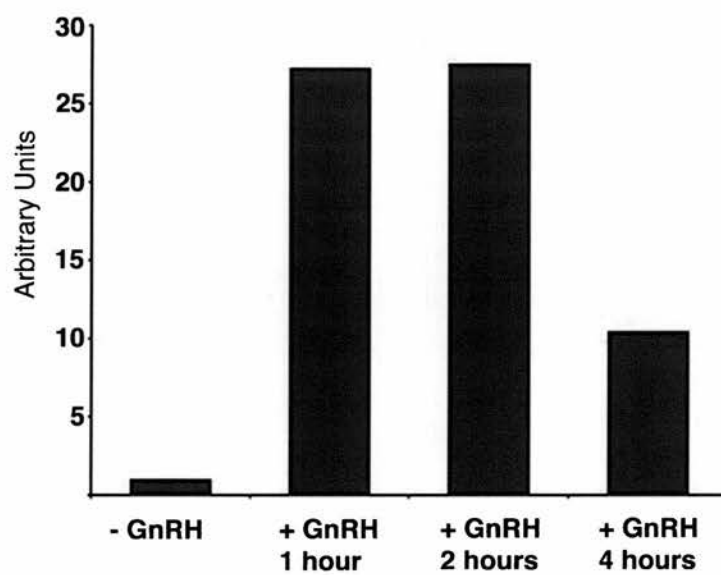


Fig 3.2.8e: Quantification of the tyrosine phosphatase mRNA expression profile.

The 18s rRNA expression was used to quantify the tyrosine phosphatase mRNA expression profile, using Imagequant (Molecular Dynamics), Excel (Microsoft), and DeltaGraph (SPSS). Expression was up-regulated 27-fold within one hour of GnRH stimulation.

FAA was identified by differential display as being regulated by GnRH and it was isolated from a differential display reaction using the R5 and T₁₂VC primers (see section 2.5.3a). The gel showed that FAA was up-regulated one hour post-GnRH treatment, began to wane after two hours, and was at basal levels after four hours post-GnRH treatment (Fig. 3.2.9a). Bioinformatics identified a match in the 43rd exon of FAA, and with a penta-zinc finger that overlaps the FAA gene between exons 35 and 43 (Fig. 3.2.9b). When the FAA clone was used as a probe in Southern blot analysis, a single 6kb hybridising band was identified (Fig. 3.2.9c), therefore the FAA clone was used to probe 40µg of total RNA on a Northern blot. Two faint bands were seen (Fig. 3.2.9d), however these bands were not informative, so an RPA was performed. The RPA confirmed that FAA was regulated by GnRH, however it showed that GnRH markedly down-regulated FAA mRNA levels one and two hours post-GnRH treatment, with mRNA levels increasing after four hours (Figs. 3.2.9e and 3.2.9f).

The results of differential display analysis of GnRH-responsive transcripts isolated from LβT2 cells are summarised in Table 3.

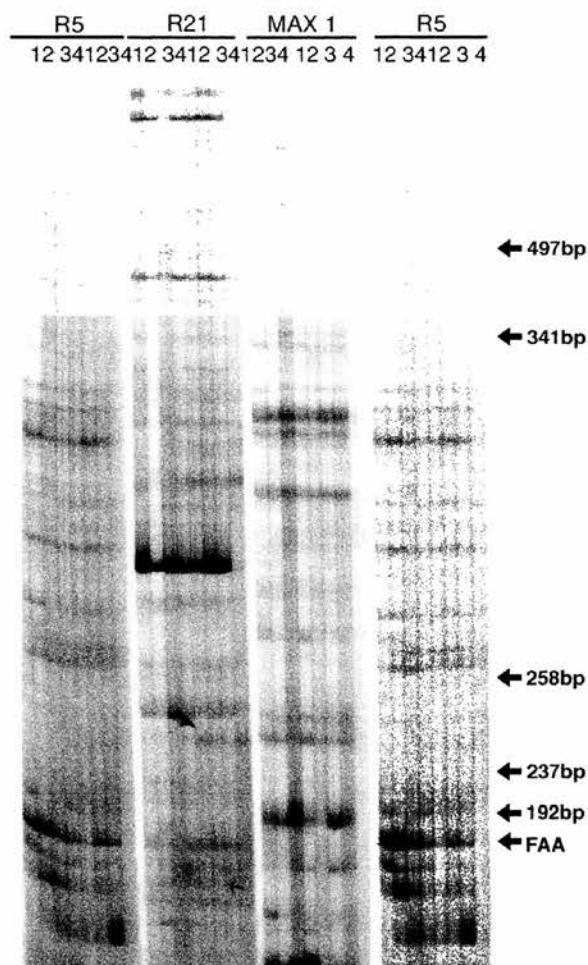


Fig. 3.2.9a: Differential display gel identifying that Fanconi's Anaemia complementation group A (FAA) mRNA is regulated by GnRH.

L β T2 cells were left untreated (lane 1) or treated with 1 pulse of GnRH and harvested: 1 hour (lane 2); 2hours (lane 3); or 4 hours (lane 4) later. Differential display was performed using T12VC anchored primers, and the random primers specified. The arrows denote the FAA mRNA expression profile, and the radiolabelled DNA size markers.

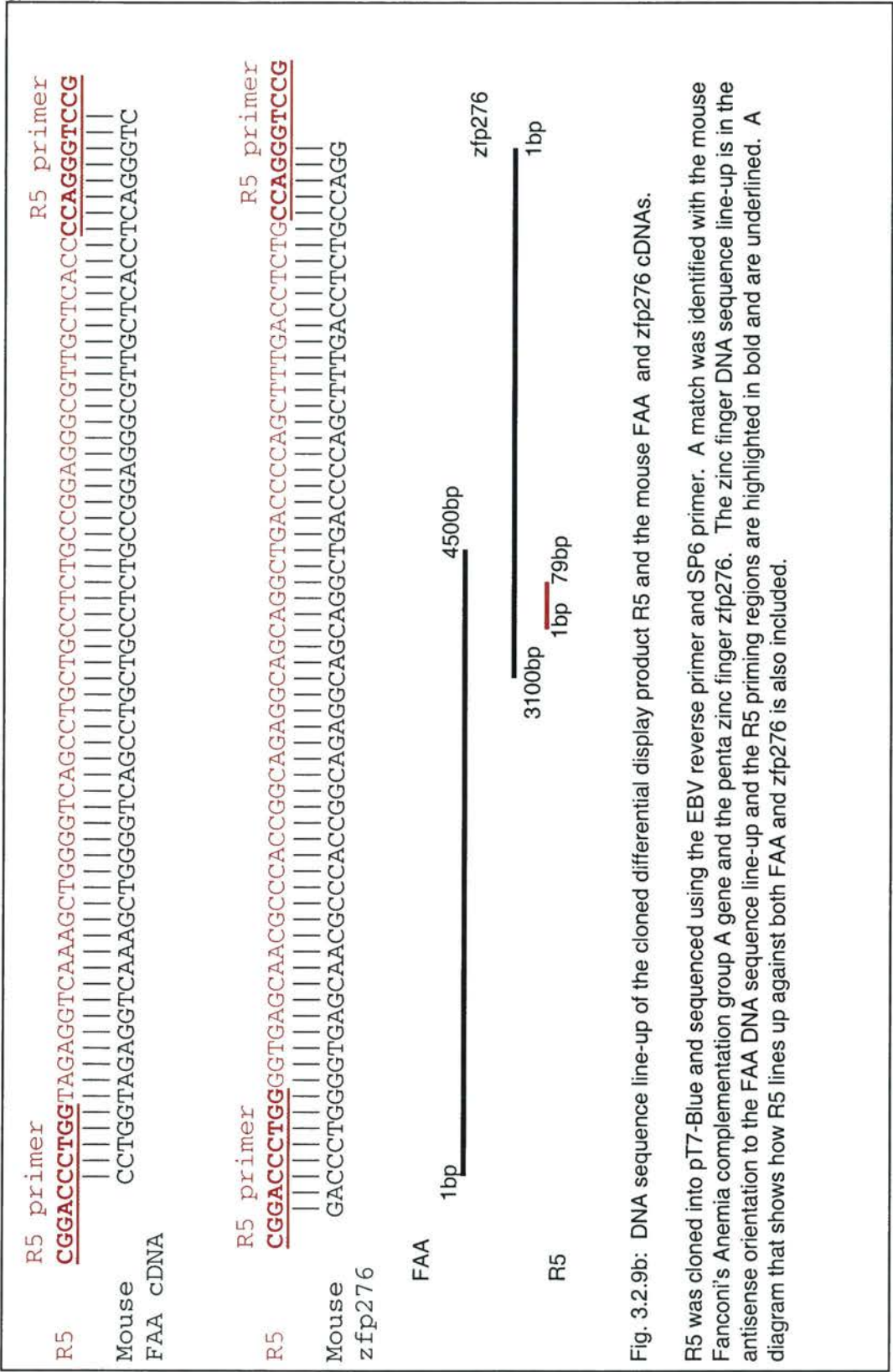


Fig. 3.2.9b: DNA sequence line-up of the cloned differential display product R5 and the mouse FAA and zfp276 cDNAs.

R5 was cloned into pT7-Blue and sequenced using the EBV reverse primer and SP6 primer. A match was identified with the mouse Fanconi's Anemia complementation group A gene and the penta zinc finger zfp276. The zinc finger DNA sequence line-up is in the antisense orientation to the FAA DNA sequence line-up and the R5 priming regions are highlighted in bold and are underlined. A diagram that shows how R5 lines up against both FAA and zfp276 is also included.

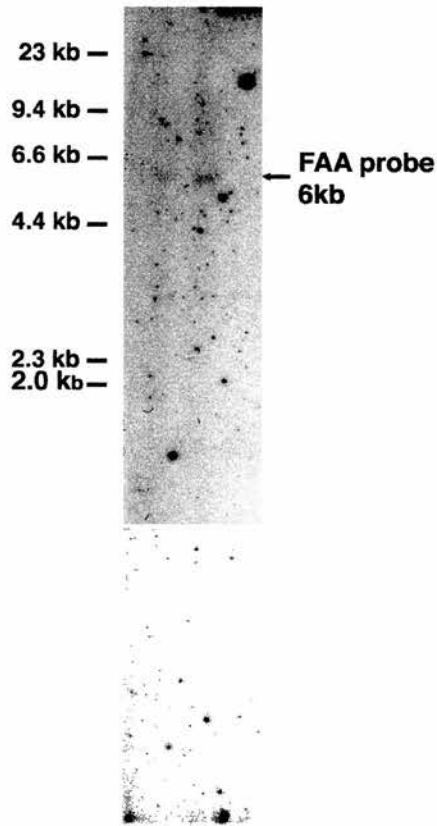


Fig. 3.2.9c: Confirmation of the suitability of the cloned differential display PCR product for use as a probe by Southern blot analysis.

Mouse genomic DNA was digested with *Bam*HI and *Hind*III, and electrophoresed on a 0.8% agarose gel, and transferred to a nylon membrane. The membrane was probed with a radiolabelled probe generated from the cloned differential display produce. DNA markers correspond to bacteriophage lambda digested with *Hind*III.

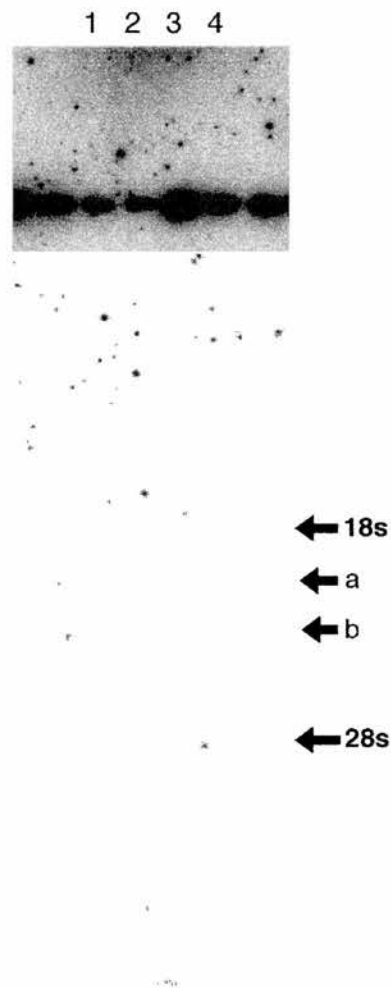


Fig. 3.2.9d: Northern blot analysis of 40µg of total RNA probed with radiolabelled FAA.

LβT2 cells were left untreated (lane 1), or treated with one pulse of GnRH harvesting at 1 hour (lane 2), 2 hours (lane 3) and 4 hours (lane 4). Total RNA was extracted from these cells, 40µg was loaded into each well, and electrophoresed on a 1% denaturing agarose gel. The RNA was transferred to a nylon membrane and hybridised with a radiolabelled FAA probe derived from the differential display clone. The arrows a and b indicate possible FAA bands, and the position of the ribosomal bands 28s and 18s are marked.

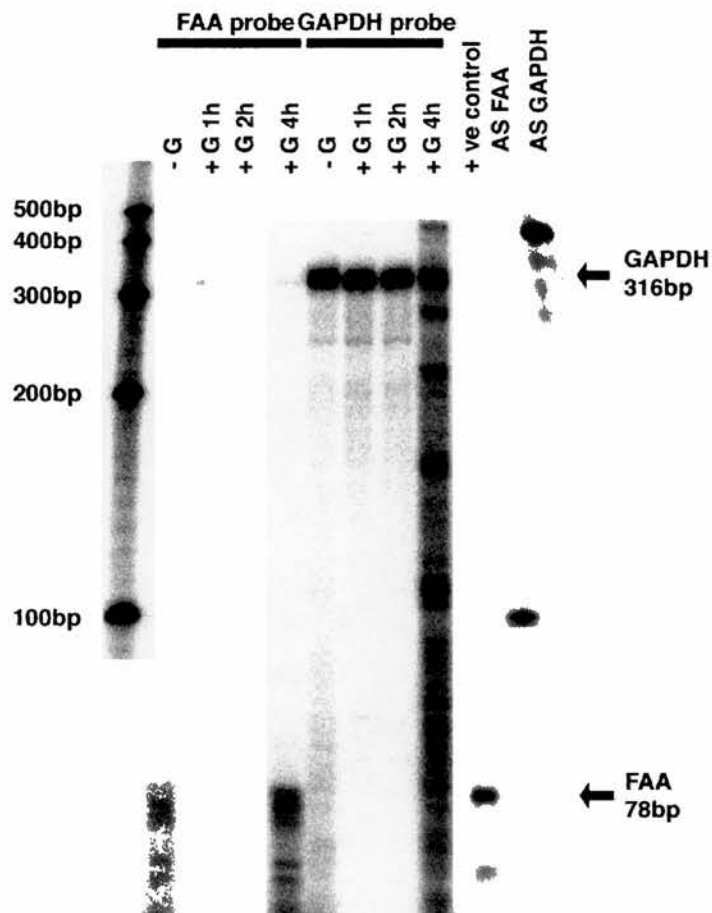


Fig. 3.2.9e: RPA analysis to confirm that FAA is regulated by GnRH.

LβT2 cells were left untreated, or treated with 1 pulse of GnRH, and harvested into lysis buffer, 1, 2, and 4 hours post-GnRH treatment. A 50μl aliquot was incubated with either 1x10⁵ c.p.m. antisense FAA probe, or with 6x10⁴ c.p.m. antisense GAPDH probe, and subjected to RPA analysis. The antisense FAA probe was also incubated with 50μl of a HeLa cell extract, which had previously been transfected with a FAA construct, this was used as a positive control. Molecular size markers and undigested antisense FAA and GAPDH probes are also indicated.

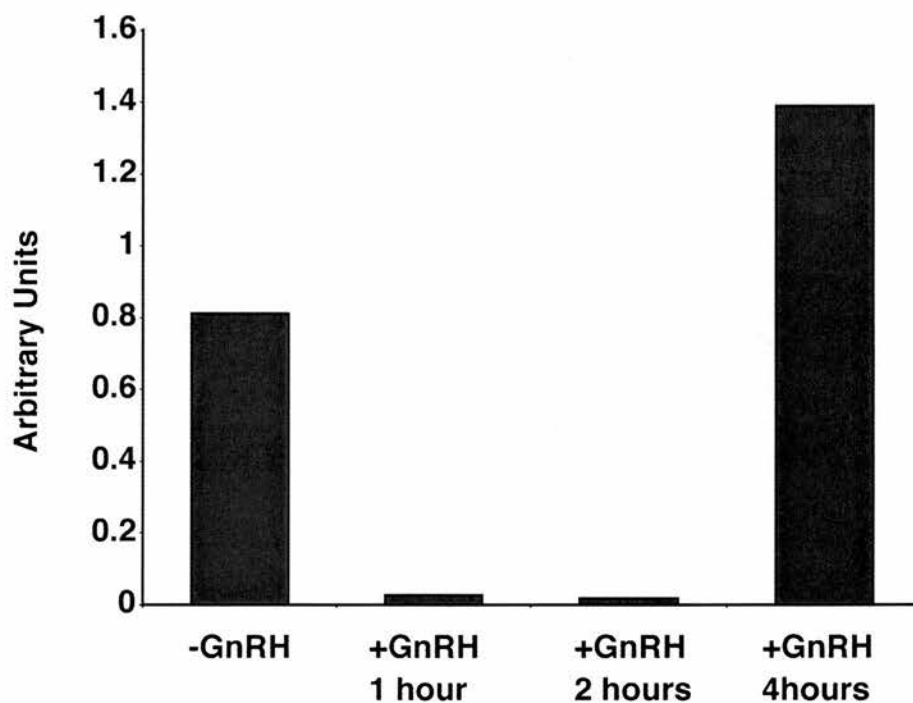


Fig. 3.2.9f: Quantification of the FAA mRNA expression profile. GAPDH was used to quantify the FAA mRNA expression profile using Imagequant (molecular Dynamics), Excell (Microsoft) and DeltaGraph (SPSS). The expression level was decreased almost to zero within one hour of GnRH stimulation.

Clone	Primers used	Size (bp)	Identity	Southern blot analysis	northern blot analysis	RPA analysis	Pattern of expression
Max1b	Max1 and T ₁₂ VA	212bp	Non-muscle myosin light chain	None	Yes: DD-RT-PCR profile was confirmed	None	Ubiquitous
R13c	R1 and T ₁₂ VC	279bp	Maps to human chromosome 3	None	Yes: No hybridising bands	Yes: No hybridising bands	May be contamination, and not a DD-RT-PCR product
R18b	R1 and T ₁₂ VC	132bp	mouse EST	None	None	None	Present in the mouse testis
R3c	R3 and T ₁₂ VC	503bp	mouse EST	Yes: Single hybridising band at 4kb	Yes: No hybridising bands detected	Yes: Faint band observed	Present in the mammary gland of pregnant or lactating mice.
R21c	R21 and T ₁₂ VC	133bp	Putative tyrosine phosphatase	Yes: Hybridising band of 4.9kb	None	Yes: confirmed the DD-RT-PCR pattern	Ubiquitous
R5	R5 and T ₁₂ VC	79bp	Fanconi's Anaemia complementation group A	Yes: Hybridising band of 6kb	Yes: But inconclusive	Yes: GnRH lowered mRNA levels	Widely expressed
Clone _d	R4 and T ₁₂ VA	105bp	Unknown	None	Hybridises to multiple transcripts	None	Unknown
Clone _c	R4 and T ₁₂ VA	103bp	Unknown	None	Hybridises to multiple transcripts	None	Unknown
R2c	R2 and T ₁₂ VC	205bp	Unknown	Yes: Hybridises to multiple transcripts	None	None	Unknown

Table 3: A summary of the DD-RT-PCR clones identified.

3.3 Discussion

3.3.1 The different pulse regimes

The first experiments were designed to mimic the luteal and follicular phases of the oestrus cycle, therefore L β T2 cells were treated with either one 15 minute pulse, or two 15 minute pulses of GnRH either 30 minutes apart (follicular phase) or 90 minutes apart (luteal phase). In this way, it was hoped that transcripts, which had a specific role in either of the two phases of the oestrus cycle would be identified. However, the mRNA expression profiles that were generated proved to be very complicated, and it was difficult to identify if the changes seen, were real or artefactual. Therefore a simpler regime, of one 15 minute pulse of GnRH, harvesting the RNA at 4 and 6 hours post-GnRH treatment, was used. The results of this regime showed that regulation of mRNA expression occurred much earlier than 4 and 6 hours; expression levels had returned to pre-GnRH treatment levels by 6 hours. So, L β T2 cells were again treated with a single pulse of GnRH, but were harvested over a shorter time course of 0, 1, 2, and 4 hours, this showed the mRNA expression levels changing rapidly over time. The most striking result of this GnRH pulse regime was that the up-regulation of mRNA levels was so rapid, a change was seen within 1 hour, which was maintained after two hours, and generally began to wane after 4 hours.

The rapidity of the cells' response suggested a mitogen activated protein kinase (MAPK) pathway was responsible for transmitting the signal from the GnRH receptor. This is because MAPK are known to phosphorylate and activate immediate early genes such as Elk-1 and serum response factor (SRF), which are constitutively bound to the serum response element of the

fos gene (Alberts, Bray et al. 1994). GnRH has been shown to stimulate MAPK in α T3-1 cells (Reiss, Llevi et al. 1997), and MAPK activity is increased within 10 minutes of GnRH stimulation (Levi, Hanoch et al. 1998). Previous evidence has suggested that the MAPK pathway transduces transcription in α T3-1 cells (Roberson 1995), and GnRH pulses are required to maintain activation of MAPK (Haisenleder, Cox et al. 1998). So, GnRH binds to and activates the GnRH-R, which activates the G protein G_q that activates phospholipase C (PLC). This leads to the formation of inositol 1,4,5-triphosphate (IP_3), and diacylglycerol (DAG) leading to elevation of Ca^{2+} and activation of protein kinase C (Alberts, Bray et al. 1994). Protein kinase C (PKC) can then activate the MAPK cascade (Han and Conn 1999).

However GnRH is also known to activate other second messenger signalling pathways. GnRH is known to activate expression of c-Jun N-terminal protein (JNK) kinase (Levi, Hanoch et al. 1998), but this activation occurs at least 30 to 60 minutes after GnRH stimulation. Since the majority of transcripts were identified as being regulated within 1 hour, this suggests that JNK may not be involved, but should be investigated. Therefore a number of signalling pathways may be involved in transmitting the GnRH signal to the nucleus, activation of which may be regulated by the GnRH pulse regime.

3.3.2 DD-RT-PCR generated transcripts containing repeat sequences that may correspond to 3' UTR

A number of the isolated transcripts contained repeat sequences, which are commonly found in 3' UTRs. These repeats proved problematic, since clones

containing repeats produced hybridising smears that were not informative. Using Southern blot analysis to screen for clones that produced distinct hybridising bands, identified those that could be used in further analysis e.g. by northern blotting or RPA. This screen enabled the straightforward clones, those lacking repeats, to be analysed first. The problematic clones, those that contained repeats, were not used. However, had time allowed, further analysis could be possible by generating full-length clones either by 5' or 3' rapid amplification of cDNA ends (RACE), which could then be used to screen mouse cDNA libraries.

3.3.3 The cloned DD-RT-PCR products were smaller than expected

All the cloned differential display products were smaller than expected, based on the size of the bands isolated from the DD-RT-PCR gels. DNA sequence analysis of the clones often identified priming sites that the upstream primer could anneal to. Since the annealing temperature of the re-amplification PCR is low (40°C), the re-amplification primers may be priming to internal primer sites, or mis-priming to sequences that have similarity to the primers themselves. Thus, the small size of the fragments may be due to internal mis-priming during the re-amplification PCR.

3.3.4 Regulation of Myosin Light Chain mRNA by GnRH

Myosin was initially identified in skeletal muscle, and is involved in the contractile mechanism of muscle fibres. There are a number of different types of myosin, the most common being myosin-II, found in skeletal

muscle fibres and smooth and non-muscle cells (Alberts, Bray et al. 1994). There are a number of smaller myosins found in all cells (the best characterised being myosin-I), which regulate vesicle transport, attach actin filaments to the plasma membrane, or align two actin filaments together, and allow them to slide past each other (Cheney and Mooseker 1992; Mooseker and Cheney 1995; Mermall, Post et al. 1998). Myosin-I consists of a single conserved motor head domain, which drives movement along the actin filaments, and a variable tail domain, which determines the role of the myosin in the cell. The tail domain of myosin-I consists of a heavy chain, and a light chain (Stryer 1995), which can be phosphorylated by myosin light chain kinase (MLCK). The activation of myosin depends on the phosphorylation of myosin light chain (MLC), by MLCK (Alberts, Bray et al. 1994). MLCK activity is Ca^{2+} dependent. Activation of PLC and PKC can induce a rise in Ca^{2+} levels. Ca^{2+} binds to calmodulin, a Ca^{2+} binding protein, which then phosphorylates MLCK, activating the kinase. MLCK then phosphorylates MLC, which activates the myosin motor (Alberts, Bray et al. 1994).

A previous paper has linked MLCK with a GnRH mediated Ca^{2+} dependent exocytosis of LH from cultured rat pituitary cells by phosphorylation of MLC (Rao, Paik et al. 1997), how MLC promotes exocytosis of the LH containing vesicles, is unknown. MLC was identified and isolated using the follicular/luteal phase pulse regime and the expression profile suggests that MLC transcription is up-regulated during the luteal phase. The northern analysis confirmed this pattern of expression, however the basal expression level was higher than the differential display profile suggested. This discrepancy may be explained by loading errors on the differential display

gel; thus the differential display expression pattern appears to show de novo synthesis, rather than an up-regulation of transcription in response to GnRH. MLC is an integral part of the cytoskeletal structure, which may also explain the high basal mRNA expression levels shown on the northern blot.

The expression profile of MLC shows that the transcriptional up-regulation of MLC mRNA was dependent on pulse frequency. This may be due to its role in secretion, and evidence for different second messenger signalling pathways involved in the control of transcription during the luteal and follicular phases. GnRH via a Ca^{2+} mediated response induces an initial spike, followed by a short sustained secretion of LH in rat pituitary cells. Wortmannin, a specific inhibitor of MLCK, abolished the sustained release of LH, indicating that this release required phosphorylation of MLC (Rao, Paik et al. 1997). However, a two pulse GnRH regime indicated that MLCK induced phosphorylation was inhibited by activation of protein kinase C (PKC) in $\alpha\text{T3-1}$ cells (Rao, Paik et al. 1997). Therefore, there may be 2 mechanisms, which mediate LH release during the luteal and follicular phases. In the luteal phase, the GnRH pulse interval is approximately one per hour, with a concomitant release of LH. GnRH induces an influx of Ca^{2+} that activates the MLCK pathway, which induces a pulse of LH to be secreted. When the GnRH pulses become more frequent, i.e. one every fifteen minutes, as in the follicular phase, the PKC pathway is activated, which inhibits phosphorylation of MLC by MLCK. This mechanism could attenuate secretion of LH, and consequently increase the number of LH containing vesicles. When the LH surge is triggered, these can be released en masse. Thus, it is hypothesised that MLC may only be required for LH

secretion during the luteal phase, and hence MLC mRNA levels are only up-regulated during the luteal phase.

3.3.5 GnRH tightly regulates tyrosine phosphatase

A putative tyrosine phosphatase was identified as being regulated by GnRH, by differential display analysis. It was massively up-regulated one hour post-GnRH treatment, and the expression waned after 4 hours post-GnRH treatment. There is evidence to suggest that GnRH binding to the GnRH receptor (GnRH-R) up-regulates transcription of a tyrosine phosphatase in pancreatic tumour cells (Liebow, Lee et al. 1990), though its role is not entirely understood. In pancreatic tumour cells that have GnRH-R, tyrosine phosphatases are activated after stimulation with GnRH, and act to suppress the tyrosine kinase response induced by epidermal growth factor (EGF) by de-phosphorylating the GnRH-R (Liebow, Lee et al. 1991). In a similar manner, the signalling cascade activated by GnRH binding to GnRH-R may likewise, regulate the GnRH response: activation of the MAPK pathway, activates the PKC pathway, which induces a number of other phosphorylation events involved in activating the GnRH response. One of these events would be to induce transcription, translation, and phosphorylation of tyrosine phosphatases, which dephosphorylate and deactivate the signalling mechanisms. In this manner, the GnRH response can be controlled, and down-regulated until the next GnRH signal. RPA analysis confirmed the expression profile and after quantification, identified the up-regulation as 27-fold. This massive up-regulation may be due to the

fact that these L β T2 cells are naïve to GnRH therefore, the cells initiate a “knee-jerk” reaction in response to GnRH, to regulate the signal pathways.

3.3.6 DD-RT-PCR identified a transcript that is found in the mammary gland, brain and CNS

The transcript R3c was identified as being up-regulated by GnRH within one hour of GnRH stimulation, which was maintained 4 hours post-GnRH treatment. Searching the mouse EST database with R3c, identified a match with an EST that was expressed in the mammary gland of a pregnant, and lactating female mouse. There was also some similarity with an EST, that localises to regions of the CNS and the brain, specifically, the hypothalamus, pituitary, corpora quadrigemina, diencephalon, spinal cord, retina, neonatal (day 0) cerebellum and cortex, and embryonic brain. These are regions of the “old brain” that control the basic survival functions such as homeostasis, breathing, heart rate, sensory and endocrine systems.

GnRH induces de novo synthesis of this transcript and expression is maintained 4 hours post-GnRH treatment. This implies that this transcript requires the presence of GnRH before it is expressed however, after the cells have been exposed to GnRH, the transcript no longer requires GnRH to be expressed. This may be an artefact of the cells, which are naïve to GnRH and hence will not express transcripts that are only GnRH responsive. This transcript is also rare, northern blot analysis could not identify any bands, therefore an RPA was performed, the results of which were inconclusive, due to degradation of the RNA. A faint band can be seen at 300bp, however

it is not possible to determine if this band is real or artefactual. Ideally, the RPA should have been repeated, but time constraints have prevented this.

3.3.7 R13c is a novel transcript that matches a region on chromosome 3, but is probably not a differential display fragment

R13c was up-regulated within one hour of GnRH treatment, expression levels peaked after two hours and waned after 4 hours post-GnRH treatment. Bioinformatics identified an exact match with a region on human chromosome 3, in the high throughput database. This, together with the lack of a hybridising band on the RPA suggests that the PCR fragment that was cloned was a result of contamination from a human source, since bioinformatic analysis produced an exact nucleotide match, when some divergence would be expected.

3.3.8 R18b has identity with an EST that is expressed in the adult mouse testis

R18b is up-regulated within one hour of GnRH exposure, this is maintained after 2 hours, but wanes after 4 hours. It matches an EST that is expressed in the adult mouse testis, however its function is currently unknown.

3.3.9 FAA is regulated by GnRH

Fanconi's Anaemia (FA) is an autosomal recessive disorder characterised by progressive bone marrow failure (Fanconi 1967). In addition, a number of

other heterogeneous clinical manifestations may be present including; short stature, limb deformities, abnormalities of major organs, skin hyperpigmentation, microcephaly, and reduced fertility or infertility (Glanz and Fraser 1982). Patients are also more susceptible to developing cancers, most frequently acute myeloid leukemia (Carreau and Buchwald 1998). This therefore suggests that the FA proteins are involved in DNA repair of crosslinking DNA strands or control of the cell cycle (Porfirio, Smeets et al. 1991; Kruyt, Dijkmans et al. 1996; Kruyt, Dijkmans et al. 1997). Complementation studies have identified seven complementation groups Fanconi's anaemia complementation group A (FAA) to G (FAG) (Joenje, Oostra et al. 1997; Joenje, Levitus et al. 2000) and mutation in any one of these genes leads to FA (Carreau and Buchwald 1998). A majority of FA patients have mutations in either FAA or Fanconi's anaemia complementation group C (FAC) (D'Andrea and Grompe 1997), which cover the entire range of possible genetic mutations (Lo Ten Foe, Rooimans et al. 1996; Savino, Ianzano et al. 1997; Lo Ten Foe, Kruyt et al. 1998; Morgan, Tipping et al. 1999; Balta, de Winter et al. 2000; Faivre, Guardiola et al. 2000). FAA mRNA was identified by differential display analysis as being up-regulated by GnRH, however RPA analysis suggests that FAA mRNA levels are down-regulated by GnRH. There may be two transcripts within this band that are both regulated by GnRH, and the transcript that appeared to be up-regulated by GnRH masked the transcript that was down-regulated by GnRH. Analysis of the EMBL database using the BLAST search tool identified an additional overlapping mRNA transcript. The zfp276 penta zinc finger mRNA transcript overlaps the 3' end of the FAA mRNA transcript in the opposite orientation (Wong, Alon et al. 2000). It is feasible that the

pattern on the differential display gel corresponds to the amplification of this transcript via priming of the downstream anchored primer and upstream priming of R5. Thus zfp276 may be up-regulated in response to GnRH. Further amplification of this cDNA prior to cloning may have generated an internal sub-clone, flanked by two R5 priming sites, which was indeed the case with this DD-RT-PCR clone (Fig3.2.9b). It is not known if this clone, and hence the DD-RT-PCR mRNA expression profile, corresponds to zfp276 or FAA (Fig. 3.3.9), this requires further clarification, but may explain the differing band profiles. Northern blot analysis of 40µg of total RNA identified very faint bands, which were not informative, but showed that FAA transcripts were not abundant in gonadotrophs. The presence of multiple bands is consistent with reports that FAA has multiple splice variants (Ianzano, D'Apollito et al. 1997), which may be involved in the regulation of FAA. The role of FAA protein has not been fully elucidated. It has been isolated in a number of different tissue types at different stages of development in mice. During mouse embryogenesis FAA mRNA expression was identified in the whisker follicles, kidneys, liver, teeth and limbs, which is consistent with the limb and major organ abnormalities seen in FA patients, and suggests a role for FAA in the development of these features. FAA expression at e13.5 was limited to the intermediate zone of the developing cerebral cortex and the anterior part of the midbrain which becomes restricted to the retina after birth (Abu-Issa, Eichele et al. 1999). FAA protein expression analysis in the adult mouse showed that it was expressed in numerous tissue types, with high levels of expression in the ovary, testis, spleen and lymphoid tissues, but no expression was seen in the brain (van de Vrugt, Cheng et al. 2000). The authors of this

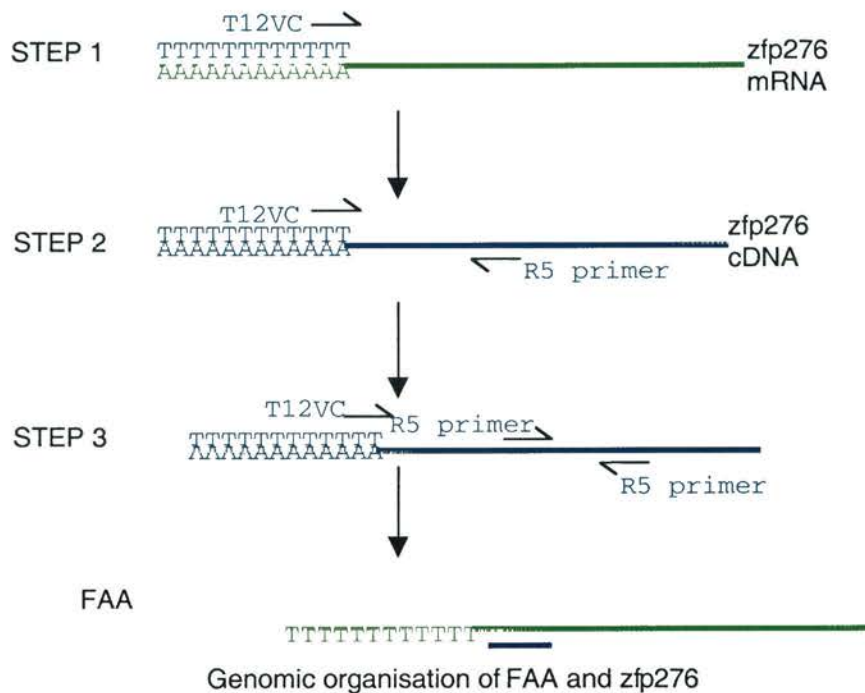


Fig. 3.3.9: Diagram to show how the R5/FAA clone was isolated, and explain why the RPA mRNA expression profile did not match the DD-RT-PCR mRNA expression profile.

The LBT2 cells were treated with GnRH and the RNA extracted. During the RT-PCR step (step 1) the 3' anchored primer annealed to the poly A tail of zfp276 mRNA producing a zfp276 cDNA. DD-PCR amplified this cDNA using the anchored primer and the R5 primer (step 2), and the DD-RT-PCR gel identified that this mRNA was up-regulated in response to GnRH. During the re-amplification PCR (step 3), the R5 primer annealed to the 5' end and to an additional R5 priming site that is present within the DD-PCR fragment, 5' to the poly A tail. This smaller fragment was preferentially amplified and cloned. The cloned fragment matches to the region of overlap between FAA and zfp276, however the orientation of the fragment was unknown. Thus RPA analysis identified that the mRNA expression levels of FAA not zfp276 were down-regulated in response to GnRH. The mRNA expression profile of zfp276 has not been confirmed.

paper however, used the whole brain. Protein and mRNA expression analysis of the different regions of the brain, including the anterior pituitary, may clarify the presence or absence of FAA in the brain. Both FAA and FAC have a role in reproduction. Recently, *FAA* and *FAC* gene disrupted mice have been reported. Both transgenic mice show chromosomal instability, however the major phenotype in the mice, appears to be reduced fertility and not anaemia (Chen, Tomkins et al. 1996; Cheng, van de Vrugt et al. 2000). Therefore, there is a role for FAA in controlling reproduction and the identification of FAA in the gonadotrophs indicates that it may be involved at both the pituitary and gonadal level of the reproductive axis. FAA's role in the gonadotrophs is explored in Chapter 4.

3.3.10 Conclusions

DD-RT-PCR has been successfully used to identify transcripts that are regulated by GnRH. Previous to the work described in this thesis, there has been evidence to suggest that two of the transcripts identified may be regulated by GnRH. MLCK activity is regulated by GnRH and is involved in regulating secretion in α T3-1 cells (Rao, Paik et al. 1997). In addition, tyrosine phosphatase activity is also regulated by GnRH in pancreatic tumour cells (Liebow, Lee et al. 1990). This thesis provides first evidence and confirms that GnRH regulates MLC and tyrosine phosphatase mRNA levels in gonadotrophs. The identification of two novel transcripts whose function is currently unknown, which are regulated by GnRH, validates the DD-RT-PCR technique. Further analysis of these transcripts may involve generating full-length clones by RACE and screening cDNA libraries, or by further bioinformatics analysis.

The expression profiles identified on differential display gels, have for the majority of cases, been confirmed by northern and RPA analysis. In general, the profiles shown by differential display are an accurate representation of the mRNA expression levels found in the cells. The contrasting DD-RT-PCR and RPA profiles of the FAA clone however, suggests that the expression profile of all clones isolated from DD-RT-PCR should be confirmed, to ensure the changes are indeed real. GnRH rapidly regulates mRNA expression levels, however this regulation is also very tightly controlled, since mRNA expression returns to basal levels four hours after GnRH treatment.

DD-RT-PCR has been very useful in identifying rare transcripts, which are GnRH regulated, however the PCR nature of this technique means that contamination from other sources may occur. Although this is a problem, the sensitivity of the technique has identified a number of transcripts whose function and identity are currently unknown, therefore it would be safe to assume that such transcripts would not have been identified by other techniques, such as cDNA arrays.

Chapter 4:

FAA regulates α GSU gene promoter activity

4.1 Introduction

Fanconi's anaemia (FA) is an autosomal recessive disorder characterised by progressive bone marrow failure and aplastic anaemia (Fanconi 1967). Other symptoms include short stature, limb deformities, abnormalities of major organs, skin hyperpigmentation, microcephaly and reduced fertility or infertility. In particular, male patients have underdeveloped gonads and defective spermatogenesis (Bargman, Shahidi et al. 1977; Glanz and Fraser 1982; Alter 1993; D'Andrea and Grompe 1997). FA patients have an increased likelihood of developing cancers, specifically acute myeloid leukemias, and squamous cell carcinomas (Dosik, Hsu et al. 1970; Swift, Zimmerman et al. 1971; Alter 1992; Somers, Tabrizi et al. 1995; Carreau and Buchwald 1998), however diagnosis is complicated by the clinical heterogeneity of the disease. The diagnosis of FA relies on the increased sensitivity of FA cells to bifunctional alkylating agents. FA cells display chromosomal instability which is amplified in the presence of crosslinking agents such as mytomyacin C (MMC) and diepoxybutane (DEB) (D'Andrea and Grompe 1997).

Complementation studies have identified seven different genes that are involved in this disorder (Joenje, Oostra et al. 1997; Joenje, Levitus et al. 2000). These were designated as Fanconi's anaemia complementation group A (FAA), B (FAB), C (FAC), D (FAD), E (FAE), F (FAF) and G (FAG), and mutations in any of these genes can cause the FA phenotype, suggesting that

they act in a common pathway (Carreau and Buchwald 1998). The nucleotide and amino acid sequences of FAA and FAC have no homology to other lower eukaryotes, however they are conserved in humans and mice (Strathdee, Gavish et al. 1992; Wevrick, Clarke et al. 1993; van de Vrugt, Cheng et al. 2000). A human homolog of FAG was identified as XRCC9, which is involved in DNA post-replication repair (de Winter, Waisfisz et al. 1998), and FAF has homology to the bacteria RNA binding protein ROM (de Winter, Rooimans et al. 2000). Furthermore, FAA interacts directly with the N-terminus of FAG (Lightfoot, Alon et al. 1999; Reuter, Herterich et al. 2000) and with FAC through the C-terminus of FAG, this forms a functional complex in the nucleus (Kuang, Garcia-Higuera et al. 2000), FAG also interacts with FAF in the nucleus. Additionally, FAE can interact with FAC directly, and with both FAA and FAG (Medhurst, Huber et al. 2001) (see Fig. 4.1).

Mutations in FAA account for 60% of all patients and include a wide range of mutation types (Wijker, Morgan et al. 1999). The *FAA* gene contains 43 exons, spans 80kb and encodes a 4.5kb mRNA transcript, with a number of alternative splice variants (Ianzano, D'Apolito et al. 1997). The FAA protein is 162kDa and has a bipartite nuclear localisation signal (NLS) at the N-terminus, which binds FAG and promotes nuclear accumulation of the FA complex (Lightfoot, Alon et al. 1999; Garcia-Higuera, Kuang et al. 2000). There are also two putative Src Homology module 2/3 domains (SH2/SH3), a partial leucine zipper domain (van de Vrugt, Cheng et al. 2000), and a peroxidase domain (Mian and Moser 1998). Unlike *FAC*, that has mutation "hotspots" that determine if the FA phenotype is mild or severe, *FAA* mutations are spread along the whole gene, and no correlation between

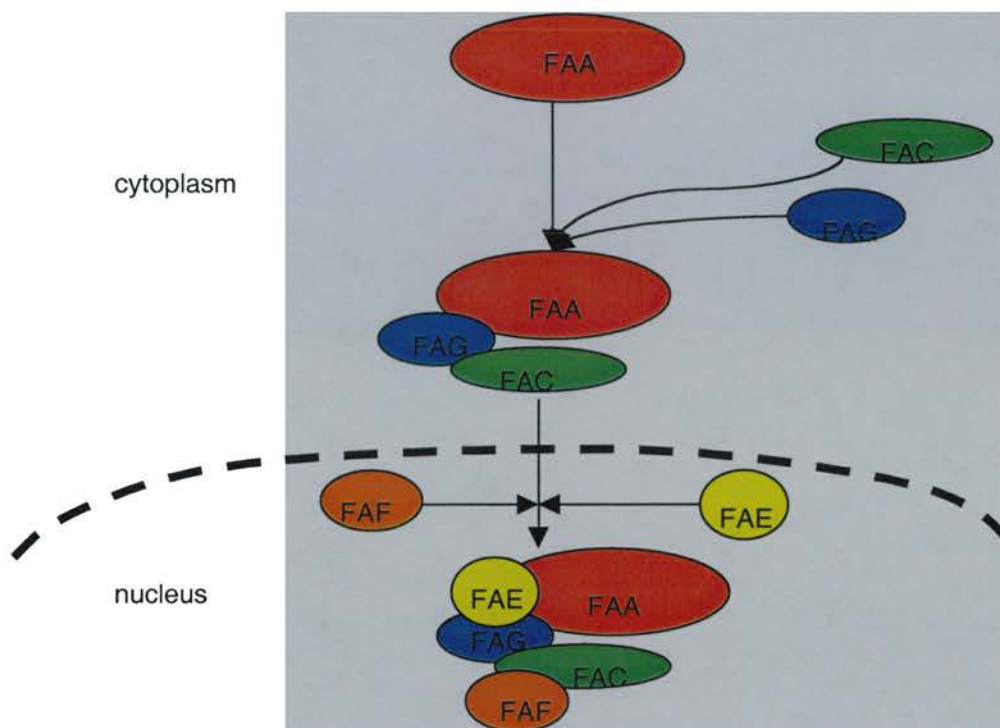


Fig. 4.1: A schematic representation of the interactions of the five known FA proteins in non-gonadotroph cells.

FAA interacts with FAG to produce a stable complex. FAC interacts with FAG and the complex is transported to the nucleus where FAF interacts with FAC, this produces a functional nuclear complex. FAE can interact with FAG and FAA, however its role is not fully understood.

mild or severe phenotypes has been observed (Carreau and Buchwald 1998). Recently, the endocrinopathy of FA patients was reviewed, and showed that FA patients had a number of endocrine abnormalities including growth hormone (GH) insufficiency, hypothyroidism, glucose intolerance, hyperinsulinism and/or overt diabetes mellitus (Wajnrajch, Gertner et al. 2001) and there was some correlation between phenotype and complementation group (Faivre, Guardiola et al. 2000). There is also evidence to suggest that a truncated FAA protein produces a more severe phenotype than the absence of a FAA protein (Chen, Tomkins et al. 1996; Cheng, van de Vrugt et al. 2000).

A *FAC* gene disrupted mouse was developed in 1996 (Chen, Tomkins et al. 1996). The mice showed no developmental or haematological abnormalities, however the mice did have inducible chromosomal instability, and reduced fertility. Homozygous matings produced no litters, and heterozygous matings produced fewer and smaller litters than their wild-type counterparts. During the course of the work in this thesis, a *FAA* gene disrupted mouse was reported (Cheng, van de Vrugt et al. 2000). Again, this transgenic mouse showed no developmental abnormalities, and only a mild haematological phenotype. The embryonic fibroblast cells of these mice did display chromosomal instability if challenged with mitomycin C and the mice were hypogonadal and subfertile.

Since the mean age of survival for FA patients is 16 years (Wijker, Morgan et al. 1999), there have been few studies on gonadotrophin synthesis and secretion. Some FA patients show subfertility, and hypogonadism (Bargman, Shahidi et al. 1977; Berkovitz, Zinkham et al. 1984), and gonadotrophin serum levels of FA patients have been reported in a pair of

siblings with FA (Berkovitz, Zinkham et al. 1984). Both patients presented with hypergonadotrophic hypogonadism, and the serum levels in both patients were significantly elevated, however the complementation group of these patients was unknown. This, together with the novel discovery of FAA in gonadotrophs using DD-RT-PCR, suggested that FAA might have a role in controlling gonadotrophin synthesis or secretion.

4.2 Results

4.2.1 Transient transfection analysis of FAA and the LH β gene promoter

Transient transfection assays were carried out in L β T2 cells, and α T3-1 cells to identify a role for FAA in gonadotrophs. In order to identify if FAA affected LH β transcription, an expression vector, containing the coding region of the FAA mRNA, was co-transfected with either -1786bp (pp1786) or -700bp (dLH2) of the ovine LH β promoter construct linked to a β -galactosidase reporter gene into L β T2 cells (see sections 2.4.9, 2.4.11 and 2.4.12). The cells were then either left untreated, or treated with GnRH 48 hours after transfection, harvested 6 hours post-GnRH treatment and assayed for β -galactosidase activity (see sections 2.4.8 and 2.4.11). The results indicated that FAA did not affect basal, or GnRH regulated LH β promoter gene activity (Fig. 4.2.1a and Fig 4.2.1b).

4.2.2 Transient transfection analysis of FAA and the α GSU gene promoter in L β T2 cells

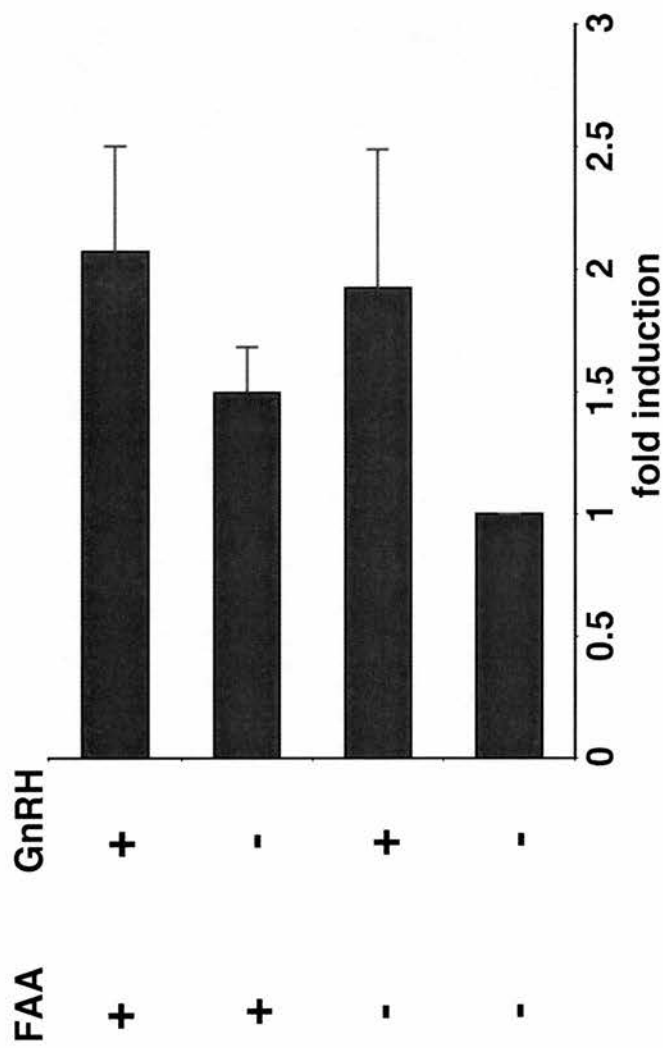


Fig. 4.2.1a: FAA has no effect on the basal, or GnRH regulated activity of a -1786bp LH β promoter in L β T2 cells. The -1786bp LH β promoter construct linked to a β -galactosidase reporter gene and a control CAT reporter gene were transfected into L β T2 cells for all treatments and FAA was either omitted (-) or added (+) to this mix. The results are expressed as fold induction after correcting for protein levels and transfection efficiency, as determined by CAT activity. After 48 hrs, the cells were either left untreated (-) or treated (+) with GnRH before harvesting. No significant differences were shown using ANOVA one-way analysis of variance.

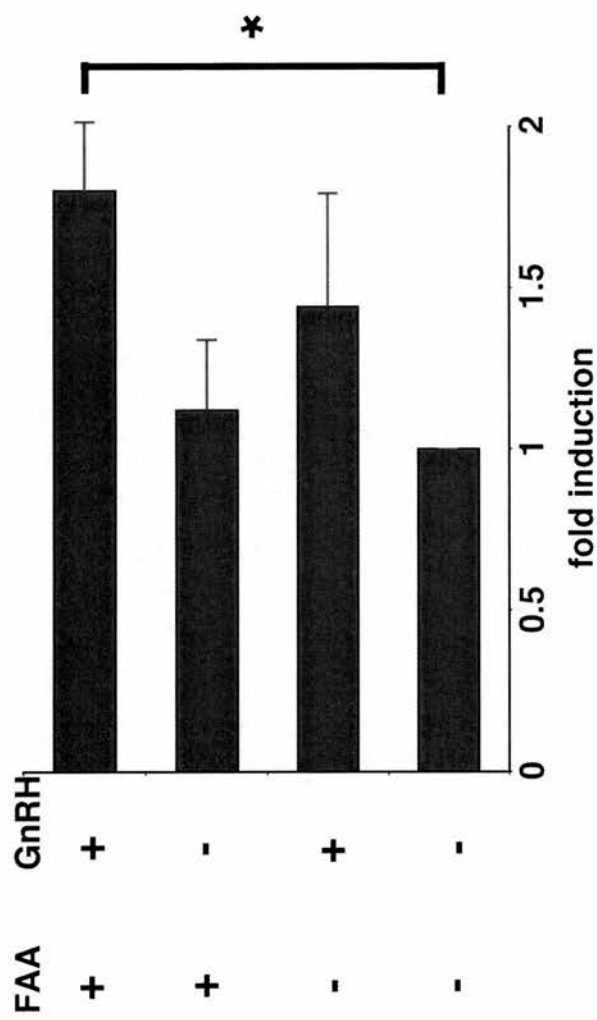


Fig. 4.2.1b: FAA has no effect on the basal or GnRH regulated activity of a -700bp LHβ promoter construct in LβT2 cells. The -700bp LHβ promoter construct linked to a β-Galactosidase reporter gene, and a control CAT reporter gene were transfected into LβT2 cells for all treatments and FAA was either omitted (-) or added (+) to this mix. The results are expressed as fold induction after correcting for protein levels and transfection efficiency, as determined by CAT activity. After 48 hours the cells were either left untreated (-) or treated (+) with GnRH before harvesting. ANOVA one-way analysis of variance was used to determine significant differences: *p< 0.05 untreated and untransfected vs. GnRH treated and transfected with FAA.

Since FAA had no effect on LH β gene promoter activity, it may have an effect on α GSU gene promoter activity and possibly explain the infertile phenotype of *FAA* gene disrupted mice. To test this, FAA was co-transfected with -480bp of the α GSU gene promoter linked to a luciferase reporter gene into L β T2 cells (see section 2.4.9). This promoter fragment is known to target α GSU expression to the gonadotroph cells of transgenic mice (Horn, Windle et al. 1992) and contains both the GnRH-RE and PGBE regions (see section 1.4.4) (Schoderbek, Kim et al. 1992) required for both basal and GnRH regulated transcription. The cells were left untreated, or treated with GnRH 48 hours after transfection, and harvested 6 hours post-GnRH treatment before assaying for luciferase activity (see sections 2.4.8 and 2.4.10).

As expected, GnRH significantly up-regulated α GSU promoter activity in L β T2 cells (Fig. 4.2.2a). However, when FAA was co-transfected there was a down-regulation of basal -480bp α GSU luciferase activity and when the cells were co-transfected with FAA and treated with GnRH, the GnRH response of the -480bp α GSU promoter was obliterated (Fig. 4.2.2a). In an effort to map the region of promoter responsible for this effect, FAA was co-transfected into L β T2 cells, with the -120bp α GSU promoter. This promoter fragment contains a paired-like homeodomain binding site, and in this case FAA significantly up-regulated the basal -120bp α GSU promoter activity above the GnRH stimulated activity and this FAA induced activity was abolished in the presence of GnRH (Fig. 4.2.2b). Clearly FAA regulates the α GSU gene promoter and may act directly or indirectly, through the paired-like homeodomain binding site.

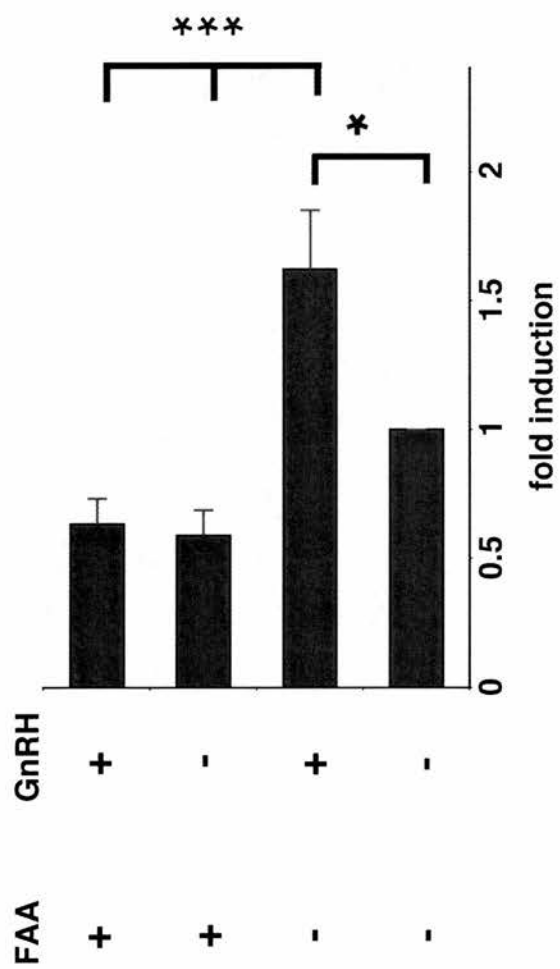


Fig. 4.2.2a: FAA represses the basal, and GnRH regulated response of the -480 α GSU gene promoter in LpT2 cells. The -480bp α GSU promoter construct linked to a luciferase reporter gene, and a control β -galactosidase reporter gene were transfected into LpT2 cells for all treatments and the cells were either left without FAA (-FAA) or co-transfected with FAA (+FAA). After 48 hours the cells were either left untreated (-) or treated with GnRH (+) before harvesting. The results are expressed as fold induction after correcting for protein, and transfection efficiency, as determined by β -galactosidase activity. Significant differences were calculated using ANOVA one-way analysis of variance. * $p < 0.05$ - FAA, untreated vs. -FAA, treated with GnRH; *** $p < 0.001$ -FAA, GnRH treated vs. untreated and GnRH treated cells, +FAA.

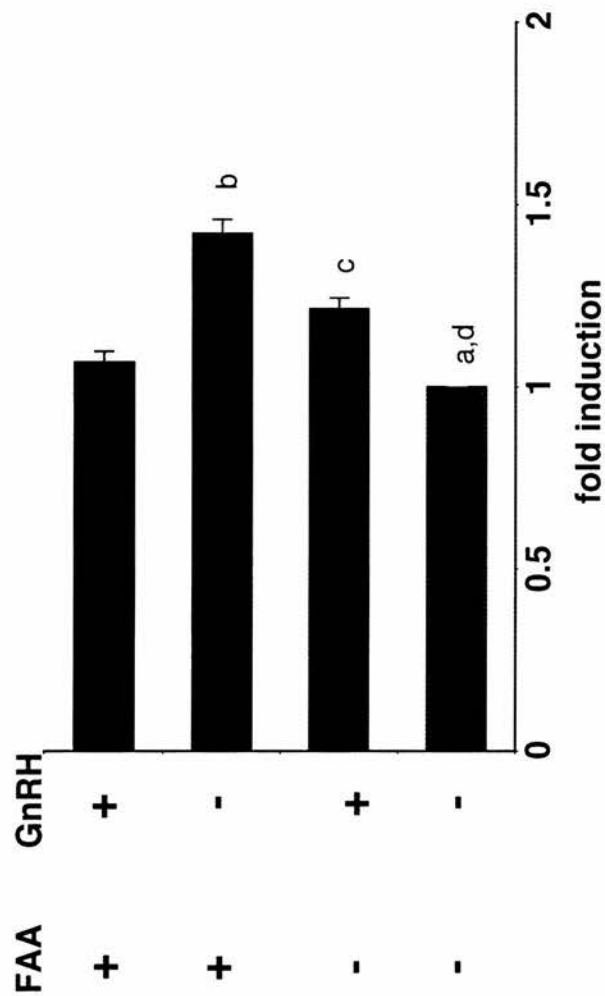


Fig. 4.2.2b: FAA up-regulates the basal activity, but represses the GnRH regulated activity of a -120bp α GSU promoter in L β T2 cells.

The -120bp α GSU promoter construct linked to a luciferase reporter gene, and a control β -galactosidase reporter gene were transfected into L β T2 cells for all treatments and the cells were either left without FAA (-FAA) or co-transfected with FAA (+FAA). After 48 hours the cells were either left untreated (-) or treated with GnRH (+) before harvesting. The results are expressed as fold induction after correcting for protein and transfection efficiency, calculated by β -galactosidase activity. Significant differences were calculated using ANOVA one-way analysis of variance: **a**, $p < 0.05$ -FAA, untreated vs. -FAA, GnRH treated; **b**, $p < 0.05$ untreated vs. GnRH treated, +FAA; **c**, $p < 0.05$ -FAA, GnRH treated vs. +FAA, untreated; **d**, $p < 0.01$ untreated vs. untreated, +FAA.

4.2.3 Transient transfection analysis of FAA and the α GSU gene promoter in α T3-1 cells

Since FAA regulated α GSU expression in L β T2 cells, it was decided to test if the same mechanism was present in α T3-1 gonadotroph cells. α T3-1 cells are of an earlier gonadotroph lineage to L β T2 cells, however they do synthesise and secrete α GSU (Windle, Weiner et al. 1990). The -480bp or -120bp α GSU promoter was co-transfected with FAA into α T3-1 cells, which were either left untreated, or were treated with GnRH 48 hours after transfection (see section 2.4.8 and 2.4.9). The cells were harvested 6 hours post-GnRH treatment and assayed for luciferase activity (see section 2.4.10). In this instance, FAA had no effect on the GnRH stimulated response of the -480bp α GSU promoter in α T3-1 cells (Fig. 4.2.3a). FAA significantly up-regulated promoter activity of the basal -120bp α GSU fragment, however GnRH did not abolish this up-regulation in α T3-1 cells (Fig. 4.2.3b). This suggests that FAA may act through the paired-like homeodomain binding site, and a factor present in both α T3-1 and L β T2 cells interacts with FAA mediating an up-regulation of the α GSU promoter activity.

4.2.4 RPA of FAA using ribo-probes generated from truncated FAA DNA constructs

Modulation of α GSU gene promoter activity indicates a role for FAA in gonadotrophin hormone production and resections of the α GSU gene promoter demonstrated that FAA could down-regulate GnRH induced gene expression over the entire promoter. Since results of DD-RT-PCR showed

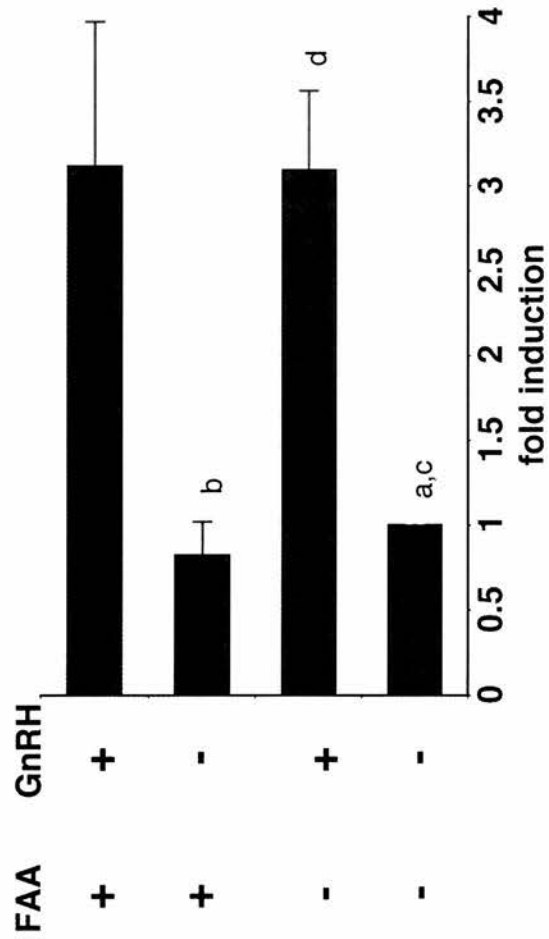


Fig. 4.2.3a: FAA has no effect on basal or GnRH regulated activity of a -480bp α GSU promoter construct in α T3-1 cells. The -480 bp α GSU promoter construct linked to a luciferase reporter gene and a control β -galactosidase reporter gene was transfected into α T3-1 cells for all treatments and either left without FAA (-FAA) or co-transfected with FAA (+FAA). After 48 hours the cells were either left untreated(-) or treated with GnRH (+) before harvesting. Results are expressed as fold induction after correcting for protein levels and transfection efficiency, as determined by β -galactosidase activity. Significant differences were determined using ANOVA one-way analysis of variance: **a**, $p<0.05$ untreated vs. GnRH treated; **b**, $p<0.05$ untreated vs. GnRH treated, +FAA; **c**, $p<0.05$ untreated, -FAA vs. GnRH treated, +FAA; **d**, $p<0.05$ GnRH treated, -FAA vs. untreated, +FAA.

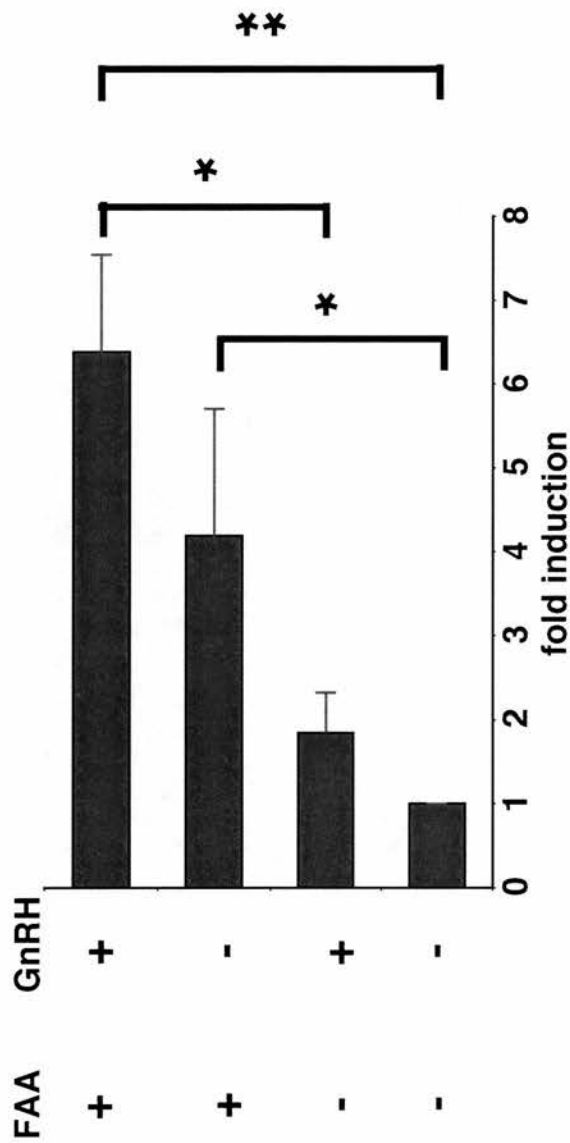


Fig. 4.2.3b: FAA upregulates both basal and GnRH regulated activity of a -120bp α GSU promoter construct in α T3-1 cells.

The -120bp α GSU promoter construct linked to a luciferase reporter gene, and a control β -galactosidase reporter gene were transfected into α T3-1 cells for all treatments and the cells were either left without FAA (-FAA) or co-transfected with FAA (+FAA). After 48 hours the cells were either left untreated (-) or treated with GnRH (+). The results are expressed as fold induction after correcting for transfection efficiency as determined by β -galactosidase activity. Significant differences were calculated using ANOVA one-way analysis of variance: * p<0.05 untreated, -FAA vs. GnRH treated, +FAA and -FAA vs. +FAA; ** p<0.01 untreated, -FAA vs. GnRH treated vs +FAA.

that the FAA mRNA was highly regulated by GnRH, it was decided to use an RPA to determine if this was due to alternative exonic splicing, or perhaps instability of the transcript. Therefore, to test this, the FAA cDNA was truncated and manipulated to produce a 965bp, and a 2400bp fragment and cloned into pcDNA3 (FAA965 and FAA2400 respectively, see sections 2.3.1 and 2.3.9 and Fig. 4.2.4a). RPA analysis was performed using antisense probes generated from these truncated forms of FAA (see section 2.3.18 and Fig. 4.2.4a). The FAA965 probe was generated by restricting the cDNA with *Eae* I, this when transcribed with Sp6 RNA polymerase generated a 375bp probe that when hybridised to FAA mRNA, would protect a 327bp hybridising band. This probe was used in a RPA analysis of L β T2 cells that were either left untreated, or treated with GnRH. There was no change in mRNA expression levels of this fragment of FAA in response to GnRH (Fig. 4.2.4b). The FAA2400 probe was also generated by restricting the cDNA fragment with *Eae* I, which generated a 399bp long probe when transcribed, that should protect a 354bp hybridising band (Fig. 4.2.4c). However, in this experiment, the control FAA mRNA generated a 175bp hybridising band, this is much smaller than expected, and requires more investigation (Fig. 4.2.4c).

4.2.5 Localisation of the FAA regulatory region

The truncated FAA cDNAs produced for the experiments described above were also used to map the region of FAA protein that mediated the down-regulation of the α GSU promoter. To facilitate this, when the cDNAs were truncated from the original full-length FAA cDNA clone, an additional

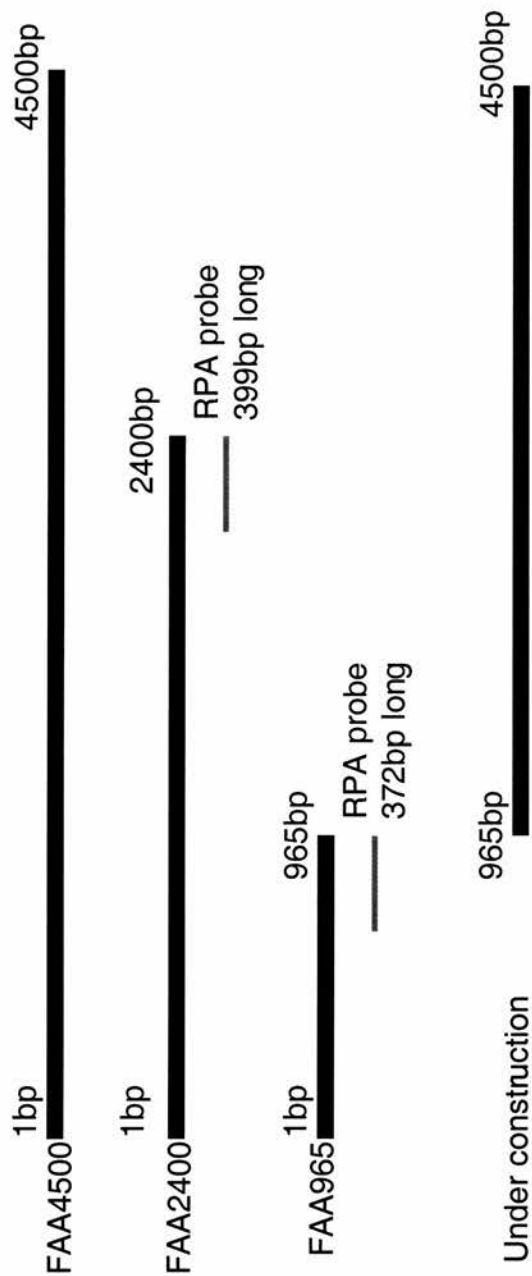


Fig. 4.2.4a: Diagram showing the truncated DNA constructs of FAA (not to scale).

The full length FAA (FAA4500) was 4500bp long. The FAA2400 construct was 2400bp long, and an antisense RPA probe of 399bp was generated from the 3' end of this construct. The FAA965 construct is 965bp long and an antisense RPA probe of 372bp was generated from the 3' end of this construct. The final construct will be 3535bp long and is under construction.

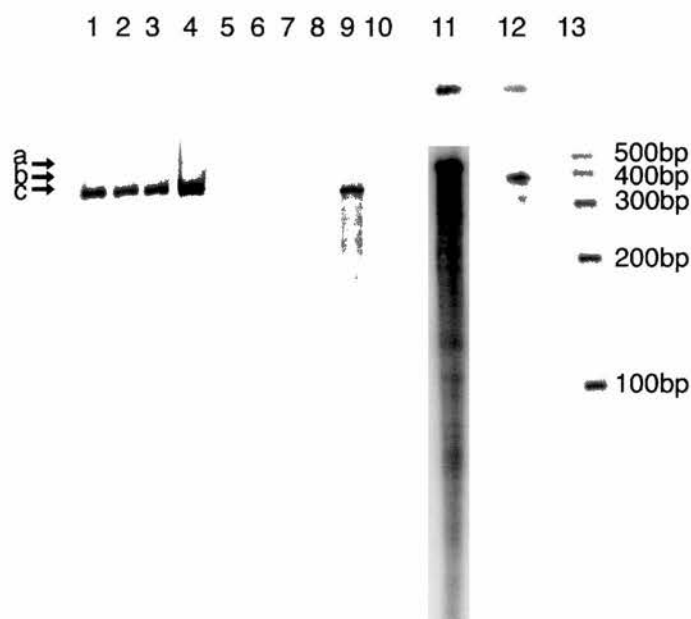


Fig. 4.2.4b: RPA analysis showing that the mRNA of the first 965bp at the 5' end of FAA is not regulated by GnRH.

LβT2 cells were either left untreated (lanes 1 and 5), or treated with one 15 minute pulse of GnRH and harvested into Direct Protect after 1 hour (lanes 2 and 6); 2 hours (lanes 3 and 7); and 4 hours (lanes 4 and 8). The cell lysate was hybridised with either radiolabelled FAA 965 (372bp) (lanes 5,6,7 and 8) or GAPDH (316bp) (lanes 1,2,3 and 4) *in vitro* translated RNA and electrophoresed on a 6% polyacrylamide gel with unhybridised FAA965 and GAPDH probes (lanes 11 and 12 respectively and arrows b and a). The FAA probe was also incubated with 20μl of a HeLa cell extract, which had previously been transfected with a FAA expression construct, this was used as a positive control (lane 9). Lane 10 is a negative control used to ensure that the FAA965 probe did not self protect, and lane 13 is a 100bp marker. Arrow c denotes the protected FAA965 (327bp) and GAPDH (316bp) bands.

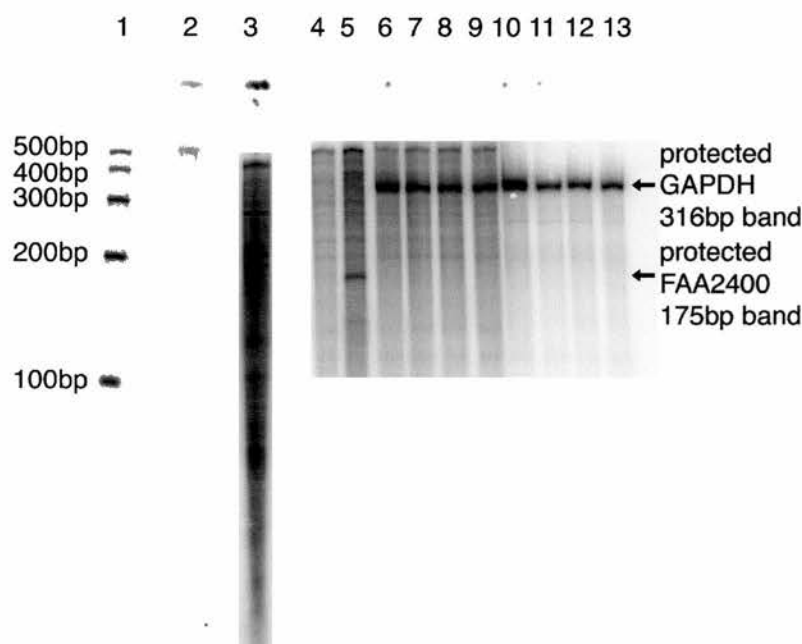


Fig. 4.2.4c: RPA analysis of the mRNA expression pattern of FAA in response to GnRH using a probe generated from FAA2400.

LβT2 cells were either left untreated (lanes 6 and 10) or treated with GnRH and harvested into Direct Protect 1 hour (lanes 7 and 11); 2 hours (lanes 8 and 12); or 4 hours (lanes 9 and 13) after treatment. The cell lysate was hybridised with either radiolabelled FAA2400 (399bp) or radiolabelled GAPDH *in vitro* translated RNA. These were then electrophoresed on a 6% polyacrylamide gel with unhybridised FAA2400 or GAPDH probes (lanes 4 and 3 respectively), and a 100bp marker (lane 1). A positive control of the FAA2400 probe hybridised to HeLa cell lysate that had previously been transfected with an FAA expression construct (lane 5), and a negative control of the FAA2400 probe hybridised to yeast tRNA (lane 2), was also included. The positive control produced a hybridising band of about 175bp.

modification of a double stranded oligonucleotide, which encoded the FLAG peptide (FLAG peptide sequence: Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-Ser-Arg-Ser), was inserted into the 3' end of the cDNA. This FLAG-tagged the proteins that were encoded by these constructs. So, the FAA965 cDNA should, when translated, produce a truncated FAA protein 322aa long and the FAA2400 cDNA should produce a protein 800aa long. In addition, the full-length FAA cDNA was also tagged by insertion of the FLAG-encoding oligonucleotide into the coding region of the cDNA. This construct encodes a 1500aa protein (Fig. 4.2.5a). Therefore, to identify the region of the FAA protein that may interact with the α GSU promoter, or mediate the repression, either FAA965, or FAA2400 or the full-length FAA cDNA construct, was co-transfected with the -480bp α GSU promoter into L β T2 cells. These cells were either left untreated, or treated with GnRH 48 hours after transfection (see sections 2.4.8 and 2.4.9). Cells were harvested 6 hours post-GnRH treatment and assayed for luciferase activity (see section 2.4.10). None of the co-transfected FAA constructs significantly affected basal α GSU promoter activity, which agrees with the result described in section 4.2.2. However, as already observed, there is a slight, but non-significant, decrease in basal expression of the α GSU promoter. When compared to the GnRH induced increase in activity of the α GSU promoter, co-transfection of all three FAA constructs significantly attenuated this response (Fig. 4.2.5b). This suggests that despite truncating the C-terminal region of the FAA protein from 1500aa down to 322aa, the remaining region could still repress. Thus, the regulatory region on FAA, lies in the region between amino acid residues 1 and 322 of the FAA protein.

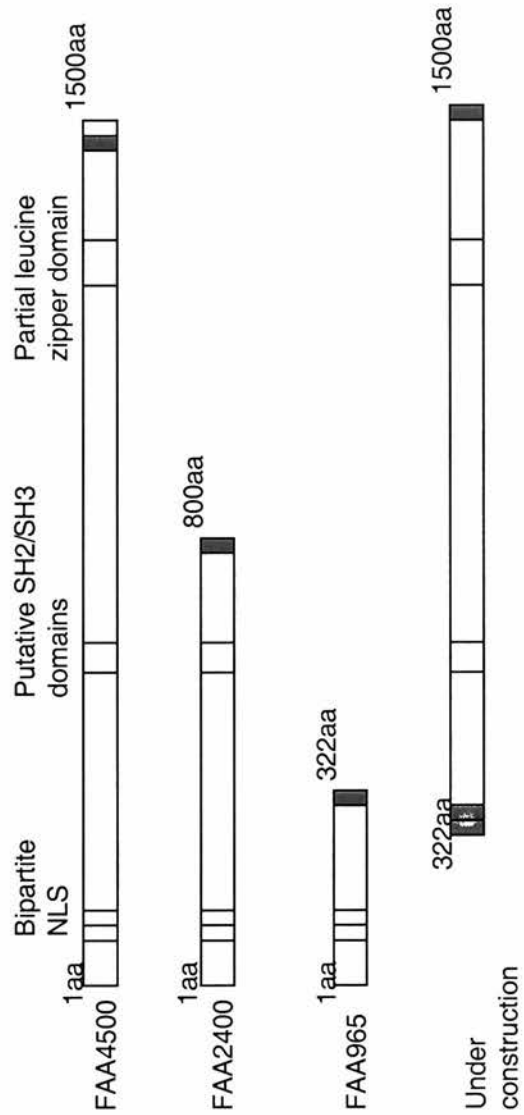


Fig. 4.2.5a: Diagram showing the truncated protein constructs of FAA.

The full length FAA contains the bipartite NLS, the 2 SH2/SH3 domains and the leucine zipper domain and is 1500aa in length. FAA2400 only contains the bipartite NLS, and the two SH2/SH3 domains and is 800aa in length. FAA965 only contains the bipartite NLS and is 322aa in length. The green boxes represent FLAG tags that have been added to the 3' end of these constructs. The final construct will have two FLAG tags on either end, an NLS signal, the two SH2/SH3 domains and the leucine zipper domain. This construct will be 1178aa in length. The putative functional region maps between 1aa and 322aa.

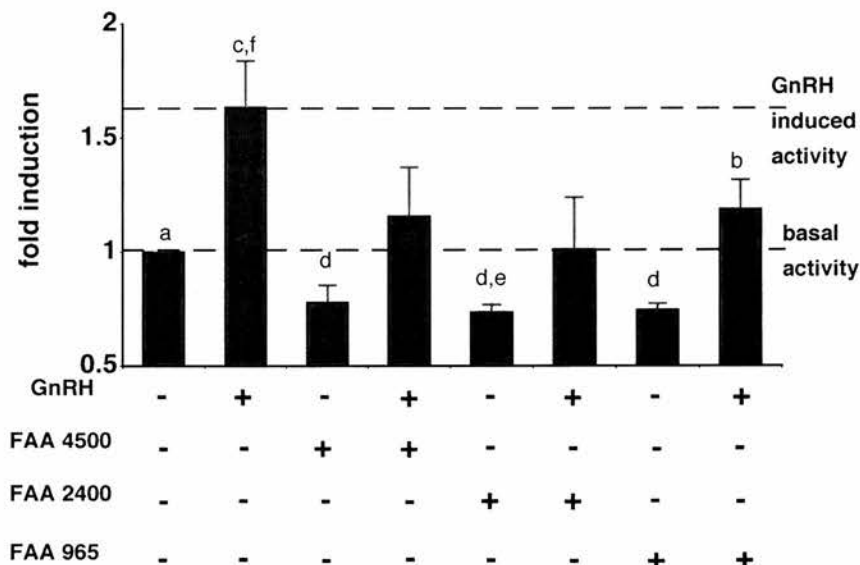


Fig. 4.2.5b: FAA965 and FAA2400 are able to repress the GnRH stimulation of the -480 α GSU promoter.

The -480bp of the α GSU promoter linked to a luciferase reporter gene and a control β -galactosidase reporter gene were transfected into L β T2 cells for all treatments and the cells were either left without FAA (-FAA) or co-transfected with FAA. The results are expressed as fold induction after correcting for protein levels and transfection efficiency as determined by β -galactosidase activity. After 48 hrs the cells were then left untreated (-) or treated with GnRH (+) before harvesting. ANOVA one-way analysis of variance was used to determine significant differences; **a**, $p < 0.01$ untreated vs. GnRH treated; **b**, $p < 0.05$ untreated, -FAA vs. GnRH treated, co-transfected with FAA965; **c**, $p < 0.01$ GnRH treated, -FAA vs. GnRH treated, transfected with FAA2400; **d**, $p < 0.001$ GnRH treated, -FAA vs. untreated, transfected with FAA4500, FAA2400 or FAA965; **e**, $p < 0.05$ untreated, transfected with FAA2400 vs. GnRH treated transfected with either FAA4500 or FAA965; **f**, $p < 0.05$ GnRH treated, untransfected vs. transfected with either FAA4500 or FAA965.

4.3 Discussion

As described in chapter 3, FAA mRNA was isolated from gonadotrophs, and was regulated by GnRH. A role for FAA in gonadotrophs was investigated by transfection studies. Initially, the effect of FAA on LH β transcription was studied. FAA was co-transfected with either -1786bp or -700bp of the LH β promoter into L β T2 cells, which were either left untreated or were treated with GnRH 48 hours later. Both LH β promoters were GnRH responsive and co-transfection of FAA did not affect this response. Therefore, FAA was co-transfected with -480bp or -120bp of the α GSU promoter into the L β T2 cell-line, a fully differentiated gonadotroph cell-type and the α T3-1 cell-line, a progenitor gonadotroph cell-type. FAA obliterated the -480bp α GSU promoter response to GnRH in L β T2 cells, and had no effect on the same promoter in α T3-1 cells. Conversely, FAA up-regulated the basal activity of the smaller -120bp α GSU promoter fragment in both L β T2 and α T3-1 cells, suggesting that they share a common factor. GnRH down-regulated this response in L β T2 cells, and had no effect in α T3-1 cells, indicating that the GnRH down-regulation of this response was specific. In an attempt to localise the functional region of FAA, two deletion constructs were produced. The leucine zipper domain of the full length FAA was removed in the FAA2400 construct, and both the leucine zipper and the SH2 and SH3 domains were removed in the FAA965 construct. Transfection studies with these constructs and the -480bp α GSU promoter in L β T2 cells mapped the repressor domain to between 1aa and 322aa.

4.3.1 FAA specifically regulates the α GSU promoter

A report in 1984 described two siblings, male and female, with FA that were hypogonadal (Berkovitz, Zinkham et al. 1984). The serum LH and FSH levels were measured in both patients and were elevated. Since FAA was identified as being GnRH regulated in gonadotrophs (see section 3.2.9), and some FA patients have been described with gonadal deformities (Bargman, Shahidi et al. 1977), it was hypothesised that FAA may have a role in regulating either the synthesis or secretion of the gonadotrophins. FAA has no effect on LH β transcription, this result is consistent with an *FAA* gene disrupted mouse that has been recently reported. The major phenotype of this transgenic mouse was hypogonadism with delayed, impaired fertility, however the mice did display a mild anaemia phenotype. Immunocytochemistry of the anterior pituitary revealed normal amounts of LH β and FSH β , suggesting that FAA does not have a role in regulating either gene (Cheng, van de Vrugt et al. 2000). However the authors did not look at α GSU protein levels, which these results suggest, should be elevated. The authors disrupted the 5' end of the *FAA* gene, specifically exons 4 to 7 and used an antibody to the 5' end of FAA to detect the protein. In the study reported in this thesis, the repressor protein domain of FAA mapped to the region deleted in the gene disrupted mice. Although it is possible that the 3' end of the protein may remain intact and account for the progressive infertility in the gene disrupted mice, it is more likely that the regulatory region of FAA required to effect α GSU gene expression was disrupted in these mice. The impaired fertility is consistent with our hypothesis that the N-terminal 322aa region of FAA was responsible for repression of α GSU, because the female gene disrupted mice produced smaller litters than their wildtype littermates and were eventually completely infertile by 20 weeks.

The male phenotype was less severe since the males were able to reproduce for longer, but the number and size of the litters declined after 20 weeks, with the majority of the adult males then failing to fertilise females. Curiously, some males regained some fertility after being infertile for some months. Histological examination of the ovaries and testes of these mice revealed that the ovaries have few follicles and the testes were atrophic with reduced spermatogenesis and hyperplasia of Leydig cells. In addition, the testes of a male FA patient also showed an increased number of Leydig cells, and a "Sertoli-cell-only" defect (Bargman, Shahidi et al. 1977). Since our studies predict that disruption of FAA would increase α GSU serum levels, it is not understood why this would impair fertility. Measurement of gonadotrophin plasma levels in the gene disrupted mice would give some indication if disrupting α GSU gene expression impacts on production of LH and FSH hormone. It is known that α GSU can be secreted from gonadotrophs into the plasma as a monomer, unbound to the β -subunit (Blomquist and Baenziger 1992), and evidence for a biological role for free α GSU is emerging (Blithe 1990; Blithe, Richards et al. 1991; Moy, Kimzey et al. 1996; Van Bael and Deneff 1996; Nemansky, Moy et al. 1998; Chabot, Magallon et al. 2000; Chabot, Gauthier et al. 2001).

Previously, a FAC gene disrupted mouse model was described with a mild haematological phenotype, and subfertility (Chen, Tomkins et al. 1996). Homozygous matings produced no litters, and heterozygous matings produced smaller litters, than a wildtype mating. The testes of the FAC transgenic mouse displayed vacuolar degeneration of the seminiferous tubules, with spermidic giant cells. The ovaries of the FAC transgenic mice were small with few follicles or mature corpora lutea. These defects may be

due to the loss of the DNA repair function in the gonads, since similar phenotypes have been seen in other DNA repair gene disrupted mice, such as the *ERCC1* gene disrupted mouse (personal communication with P. T. K. Saunders). The similarities of the gonadal phenotypes of *FAA* and *FAC* gene disrupted mice suggest that both *FAA* and *FAC* interact in the gonad. The phenotype of the *FAA* and *FAC* gene disrupted mice also share some similarity with the gonadal phenotype of transgenic mice and human patients with defects in gonadotrophin subunit or gonadotrophin receptor (Berthezene, Forest et al. 1979; Burns, Yan et al. 2001; Lei, Mishra et al. 2001). It is not certain in these mice however, if there is also a defect in α GSU synthesis, or viable gonadotrophin production. The results of the study in this thesis shed some light on the role of the FA proteins on gonadotrophin synthesis, since the regulatory domain of *FAA* was mapped to the region that interacts with *FAG*, and *FAC* (Lightfoot, Alon et al. 1999). This may mean that although the regulatory domain was mapped on *FAA*, this domain actually corresponds to the region that interacts with *FAG* and *FAC*. At the moment it is unknown if *FAG* and *FAC* are expressed in the gonadotroph. However, it is known that *FAG* interacts with *FAA* via the NLS in other cell types and it is possible that the same mechanism occurs in gonadotrophs.

4.3.2 *FAA* may interact through the paired-like homeodomain binding site on the α GSU promoter

FAA repressed the longer -480bp α GSU promoter in a cell-specific manner, but not the shorter -120bp region. It was established that -480bp of the

α GSU promoter was sufficient to target a β -galactosidase reporter to the gonadotrophs of a transgenic mouse, so this region was necessary and sufficient for α GSU transcription *in vivo* (Horn, Windle et al. 1992). Analysis of this region has identified a number of transcription factor binding sites, which can be broadly categorised into two regions, a GnRH regulated region (GnRH-RE), and a pituitary glycoprotein basal element (PGBE) (Schoderbek, Roberson et al. 1993). Currently it is known that Lhx-2, p-Lim and an Ets factor bind to the GnRH-RE and up-regulate GnRH regulated transcription (Roberson, Schoderbek et al. 1994; Bach, Rhodes et al. 1995; Roberson 1995: Glenn, 1999 #196). DNA sequence analysis has also identified the binding sites that make up the PGBE, which include SF-1, Sp1, GATA, and a paired-like homeodomain binding site (Schoderbek, Roberson et al. 1993; Barnhart and Mellon 1994; Tremblay, Marcil et al. 1999). Since repression of the -480bp α GSU promoter is specific to L β T2 cells, it would suggest that FAA can interact with a factor that is unique to fully differentiated gonadotrophs. α T3-1 cells do not appear to have this factor despite being able to synthesise and secrete α GSU, this may be because these cells are representative of an earlier stage of development of the gonadotrophs (Windle, Weiner et al. 1990).

The -120bp α GSU promoter contains a paired-like homeodomain binding site, which can bind homeobox proteins such as Pitx1, and Otx (Acampora, Mazan et al. 1998; Treier, O'Connell et al. 2001) and FAA may act through the paired-like homeodomain binding site to up-regulate expression levels. A similar homeodomain binding site is present in the LH β promoter, and Pitx1 binds to this region, to up-regulate LH β transcription (Tremblay, Lanctot et al. 1998; Quirk, Lozada et al. 2001). There is a single base-pair difference

between the paired homeodomain region on the LH β promoter and the one on the α GSU promoter (Tremblay, Lanctot et al. 1998). However FAA is not a homeobox protein, therefore FAA may interact with a homeobox protein that specifically binds to this region on the α GSU promoter. Since FAA up-regulates the -120bp α GSU promoter in both L β T2 and α T3-1 cells, a common factor that interacts with FAA, possibly through the homeodomain binding site, is present in both cell types. This may be Pitx1 or Pitx2, an isoform of Pitx1, which when mutated causes Reiger syndrome (Lu, Pressman et al. 1999) and is postulated to bind to this homeodomain binding site.

Recent DNA sequence analysis of FAA has identified a penta zinc-finger protein in an antisense orientation that overlaps, tail to tail, the 3' UTR and exons 40 to 43 of the FAA gene (Wong, Alon et al. 2000). Zinc finger proteins are involved in transcriptional regulation through sequence specific binding to DNA, RNA and through protein-protein interactions (Stanojevic, Hoey et al. 1989; el-Baradi and Pieler 1991; Seto, Lewis et al. 1993). Since this zinc finger protein is co-expressed with FAA, it may interact with FAA to regulate its action in other cell types, however it is not known if this protein is expressed in gonadotroph cells. There have been previous reports of antisense RNA interactions, which regulate gene expression and translation in *Xenopus*, *Dictyostelium*, and *C. elegans* (Kimelman and Kirschner 1989; Hildebrandt and Nellen 1992; Lee, Feinbaum et al. 1993), therefore levels of these transcripts may be controlled by a mutual antisense regulatory mechanism.

4.3.3 The region that represses the α GSU promoter was mapped to the N-Terminal 322aa

Although the *FAA* gene has been cloned, it has not been fully characterised, certainly not its function in gonadotrophs, but some information is available on how FAA may function in other cell-types. Reports have suggested that FAA contains a bipartite nuclear localisation signal (NLS) at the N-terminus of the FAA protein; two putative Src homology modules 2 and 3 (SH2/SH3) domains; a peroxidase domain; and a partial leucine zipper (Mian and Moser 1998; van de Vrugt, Cheng et al. 2000). In order to identify the repressor region, the FAA cDNA was truncated at 965bp (FAA965), and 2400bp (FAA2400). FAA2400 contained the NLS and the SH2/SH3 domains, but not the leucine zipper domain, and FAA965 only contained the NLS region. Co-transfection with the -480 α GSU promoter identified that both truncated proteins repressed the GnRH response as efficiently as the full-length protein when compared to the GnRH response of the α GSU promoter in the absence of FAA.

FAA exists both in the nucleus and the cytosol, indeed functional activation of FAA requires it to form a complex with FAC, FAG and FAF (Naf, Kupfer et al. 1998; Garcia-Higuera and D'Andrea 1999; Garcia-Higuera and D'Andrea 1999; Kupfer, Naf et al. 1999; Waisfisz, de Winter et al. 1999; de Winter, van der Weel et al. 2000). Yeast 2-hybrid analysis has identified a strong interaction between FAA and FAG, and other evidence suggests that the FAA/FAG interaction is necessary and required for transporting FAA into the nucleus (Naf, Kupfer et al. 1998; Kruyt, Abou-Zahr et al. 1999; Reuter, Herterich et al. 2000). FAG was identified as XRCC9, a protein involved in

DNA post-replication repair and cell cycle checkpoint control (de Winter, Waisfisz et al. 1998). The bipartite NLS is functional in FAA, it is sufficient but not essential for nuclear localisation, however the N-terminus region of FAG is required to bind the NLS of FAA to ensure that FAA is translocated to the nucleus (Kuang, Garcia-Higuera et al. 2000). Mutation of the NLS impedes transport into the nucleus but does not prevent it. Additionally, mutant forms of FAA with deletions of the C-terminal 70 or 260 amino acids localise FAA to the cytoplasm (Lightfoot, Alon et al. 1999). This suggests that a number of regions on FAA are involved in transporting it into the nucleus, and that FAA shuttles between the nucleus and the cytoplasm.

FAC also has functional significance and complexes with FAA to impart activity (Yamashita, Kupfer et al. 1998), however it does not bind FAA directly, instead the C-terminus of FAG is required to bind FAC and form a functional complex in the nucleus (Kuang, Garcia-Higuera et al. 2000; Reuter, Herterich et al. 2000). The FAC protein can interact with a number of different proteins with different functions and is localised to the cytoplasm and, as part of the FA protein complex, in the nucleus (Kupfer, Naf et al. 1997; Hoatlin, Christianson et al. 1998; Kruyt and Youssoufian 1998). A novel transcriptional repressor protein that contains a broad complex, tramtrack and bric a brac/pox virus and zinc finger (BTB/POZ) domain, named Fanconi anemia zinc finger protein (FAZF) interacts with FAC (Hoatlin, Zhi et al. 1999). FAZF is homologous to the promyelocytic leukemia zinc finger (PLZF) which acts as a repressor by recruiting co-repressors such as N-CoR or Sin3 (David, Alland et al. 1998; Huynh and Bardwell 1998). PLZF represses transcription of specific targets by recruitment of histone deacetylase through the SMRT-mSin3-HDAC corepressor complex (David, Alland et al.

1998) and mutations in this protein lead to acute promyelocytic leukemia reminiscent of the FA phenotype (Melnick and Licht 1999). BTB/POZ containing proteins are involved in oncogenesis (Baron, Nucifora et al. 1993; Chen, Zelent et al. 1993; Chen, Brand et al. 1993), haemtopoeisis (Ye, Cattoretti et al. 1997) and limb development (Grimm, Sporle et al. 1999). Therefore, a mutation in FAC that prevents it from interacting with FAZF could conceivably lead to the symptoms shown by FA patients. Additionally a mutation in FAA that prevents FAC from interacting in the nucleus, leading to a non-functioning complex, would also have the same outcome. FAC can also interact with Grb94 to regulate its intracellular expression levels and hence its activity (Hoshino, Wang et al. 1998). Grb94 is an adaptor protein that interacts with SH2/SH3 domains and receptors to transduce signals from the receptor to the second messenger signalling pathways (Cowan and Henkemeyer 2001). FAC can also bind and facilitate the activation of STAT1 by gamma interferon and haematopoietic growth factors by controlling the trafficking and docking of STAT1 to the gamma interferon receptor (Pang, Fagerlie et al. 2000). It also interacts with heat shock protein 70 (Hsp70) (Pang, Keeble et al. 2001) and with glutathione S-Transferase P1-1 (GSTP1) to increase GSTP1 activity (Cumming, Lightfoot et al. 2001), and both pathways prevent apoptosis in haematopoietic cells. Therefore the bone marrow failure and anaemia seen in FA patients may be due in part, to excessive apoptosis of the haematopoietic cells.

4.3.4 The FA proteins may interact in a transcription coupled DNA repair pathway

The FA protein complex can interact with BRCA1, a tumour suppressor gene linked to familial breast and ovarian cancers (Hall, Lee et al. 1990; Garcia-Higuera, Taniguchi et al. 2001). BRCA1 is involved in ionising radiation and oxidative damage repair to DNA (Gowen, Avrutskaya et al. 1998). It associates with the DNA repair proteins Rad50 and Rad51 in a mutually exclusive manner to induce homologous recombination in DNA double strand breaks (Zhong, Chen et al. 1999). Rad50 complexes with Mre11, p95 and BRCA1 and is responsible for end processing and Rad51 is involved in strand exchange at a later stage. BRCA1 is also required for transcription coupled repair (TCR) of oxidative DNA damage (Gowen, Avrutskaya et al. 1998). TCR is a process that repairs UV and oxidative induced damage on transcriptionally active DNA faster than the genome as a whole (Hanawalt 1994). It requires an active RNA polymerase II, which also acts as a DNA damage recognition complex to the DNA repair complex that is tethered to it, therefore repair is carried out concurrently with transcription (Leadon and Lawrence 1991; Christians and Hanawalt 1992). BRCA1 binds to RNA polymerase II and several transcription factors including TFIIF, TFIIE and TFIIH, and acts in concert with DNA repair enzymes during periods of rapid growth (Scully, Anderson et al. 1997; Scully, Chen et al. 1997). BRCA1 can interact with the FA proteins in a common pathway, and may provide a link between DNA repair and FA (Garcia-Higuera, Taniguchi et al. 2001). The interaction appears to be mediated through FAD2. The FAD complementation group consists of two genes *FAD1* and *FAD2*, which function downstream of the FA nuclear complex since the nuclear complex can form in FAD deficient cells (Yamashita, Kupfer et al. 1998; Timmers, Taniguchi et al. 2001). Activation of FAD2 requires the protein to be mono-

ubiquitinated, this occurs during S-phase and during DNA damage and only in the presence of the FA nuclear complex and BRCA1 (Garcia-Higuera, Taniguchi et al. 2001). FAD2 is highly conserved among lower eukaryotes including *Arabidopsis*, *Drosophila*, and *C.elegans* (Timmers, Taniguchi et al. 2001) and appears to be the first described downstream effector of the FA nuclear complex. However the BRCA1 null mouse does not display any of the FA characteristics, which implies that the FA proteins interact in a separate pathway (Garcia-Higuera, Taniguchi et al. 2001).

4.3.5 A region in FAA has homology to Ste5, a yeast MAPK scaffold protein

The current literature suggests that the FA proteins act as a complex involved in DNA repair and checkpoint control of mitosis, however gonadotroph cells do not undergo mitosis, so what is FAA doing in gonadotrophs? It is uncertain if the other FA proteins (B, C, D, E, F, G) are present in these cells, so FAA may have either a similar or a completely different role in gonadotrophs. Lightfoot et al identified a region between aa residues 36 and 250 that was crucial for nuclear localisation. FAG binds to the NLS region adjacent to this region, which is also involved in localising FAA to the nucleus (Lightfoot, Alon et al. 1999; Kuang, Garcia-Higuera et al. 2000). This region also has significant homology with the molecular scaffold protein Ste5, which is involved in the pheromone mating response in *S. cerevisiae* (Mahanty, Wang et al. 1999). In addition, this is the region that was deleted in the FAA gene disrupted mice and shown to be pivotal in reproduction. Ste5 is a molecular scaffold, which in the absence of the yeast mating type

pheromone factor, resides mainly in the nucleus and in the presence of pheromone, is translocated from the nucleus to the plasma membrane (see Fig 4.3.5a). Pheromone binds to and activates a G-protein coupled receptor (GPCR) and additionally pheromone and GnRH are functionally conserved. Therefore these similarities suggest that FAA and Ste5 may function in a similar manner, this is conjecture, but forms the basis for a working hypothesis of the role of FAA in gonadotrophs. Ste5 functions by binding proteins of the MAPK pathway and translocating them to the plasma membrane in response to pheromone. Thereafter, the G β subunit transduces the pheromone signal by binding Ste20, a MAPKKKK (Leeuw, Wu et al. 1998). Ste20 then activates Ste11, which activates Ste7, which activates the MAPKs Fus3 and Kss1. These kinases can then activate other genes involved in the mating response (Gustin, Albertyn et al. 1998). Ste5 tethers these MAPK kinases in the presence and absence of pheromone, and facilitates their activation *in vivo* by maintaining them in close proximity (Choi, Satterberg et al. 1994; Kranz, Satterberg et al. 1994). This tethering also provides specificity of this pathway to the mating response, since these MAPKs are also involved in other signalling responses (Roberts and Fink 1994; Yashar, Irie et al. 1995; Posas and Saito 1997). In mammals, β -arrestin and JIP1 have also been suggested as scaffold proteins that facilitate JNK signal transduction through GPCRs and the mixed lineage protein kinase (MLK) group respectively (Dickens, Rogers et al. 1997; Ito, Yoshioka et al. 1999; McDonald, Chow et al. 2000; Miller and Lefkowitz 2001). β -arrestins

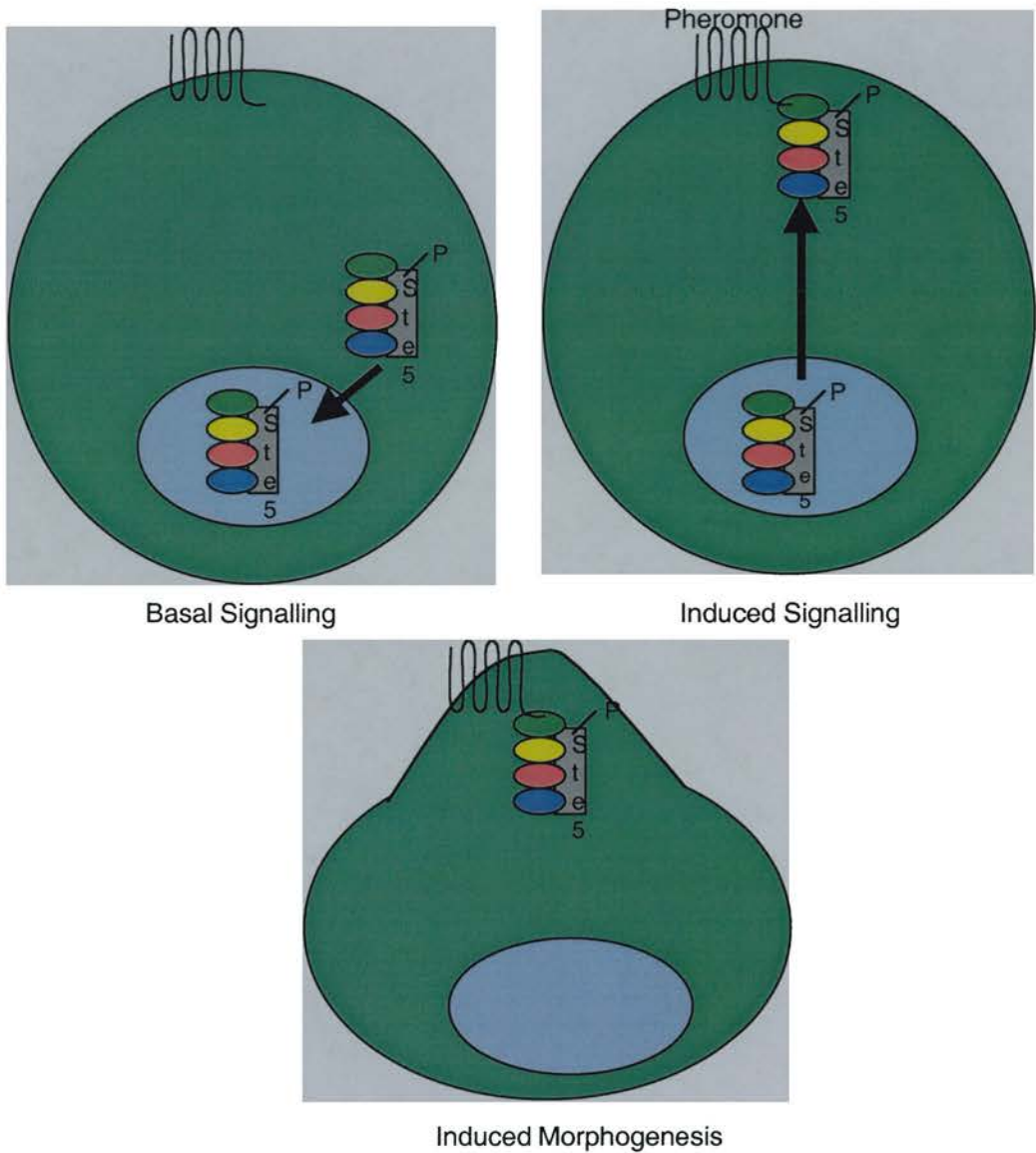


Fig. 4.3.5a: Diagram showing how Ste5 shuttles from the nucleus to the membrane in response to the pheromone signal.

Ste5 resides mainly in the nucleus in a phosphorylated form. When pheromone activates the G-protein coupled receptor, Ste5 shuttles from the nucleus to the plasma membrane and interacts with the receptor. The receptor can then activate the MAPK proteins attached to Ste5 and activate the signalling mechanisms to induce morphogenesis.

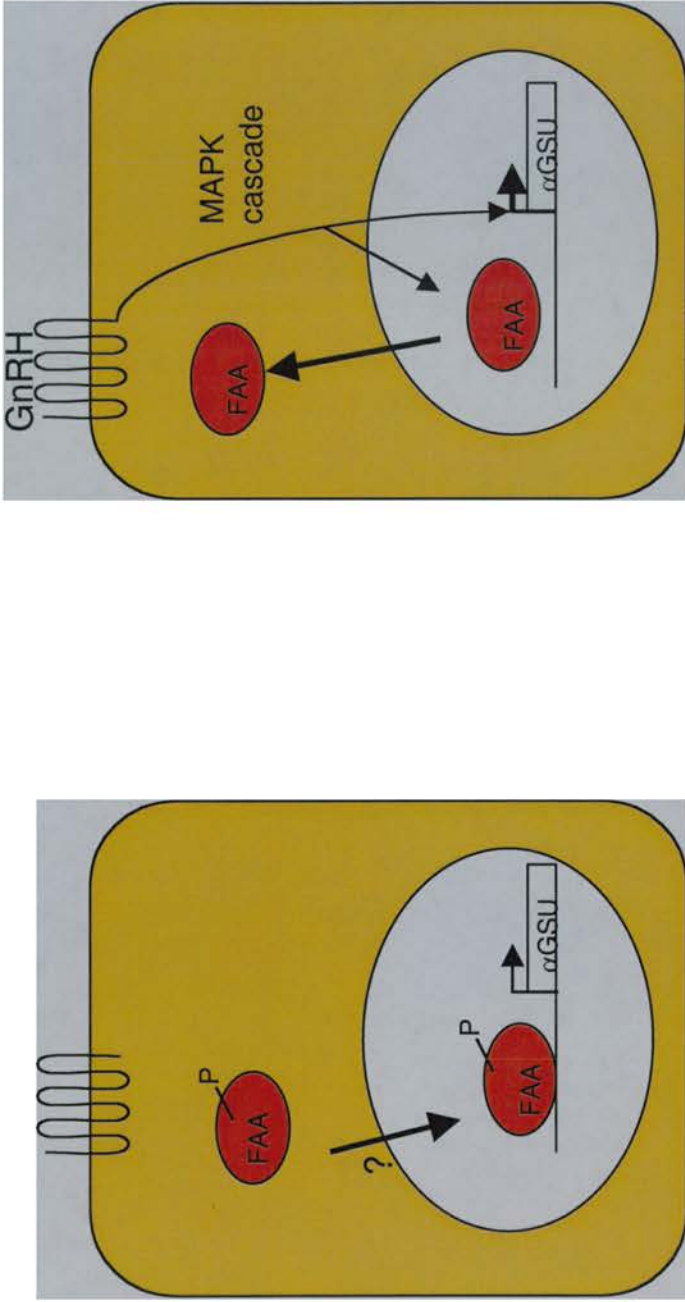


Fig. 4.3.5b: A schematic representation of the hypothesised role of FAA in regulating α GSU transcription. Phosphorylated FAA is transported into the nucleus and interacts with the α GSU promoter, either directly or indirectly. Therefore α GSU transcription is maintained at a basal rate. The GnRH receptor is activated when GnRH binds. It then activates second messenger signalling pathways, which can up-regulate the factors involved in GnRH regulated transcription and activate a phosphatase that dephosphorylates FAA. Thus FAA is transported from the nucleus, and α GSU transcription is up-regulated.

bind to the cytoplasmic tail of GPCRs and acts as a site of assembly for a functional JNK signalling module (McDonald, Chow et al. 2000), but are not able to bind the GnRH-R (Heding, Vrecl et al. 2000; Vrecl, Heding et al. 2000), suggesting that a different mechanism must be used.

Although FAA does not appear to bind second messenger signalling molecules, it may have a similar role as Ste5. So, it is hypothesised that in the non-GnRH stimulated state, FAA (or the FA nuclear complex) resides in the nucleus in a phosphorylated state, bound either directly or indirectly to the α GSU promoter. It may interact with a co-repressor or with factors that are involved in up-regulating α GSU transcription, thus controlling basal α GSU transcription. When GnRH is present, the FAA protein (or complex) is dephosphorylated and transported from the nucleus, to the cytoplasm, thus allowing GnRH stimulated up-regulation of α GSU transcription (Fig. 4.3.5b). Since mammalian GnRH receptors do not have a cytoplasmic tail, it is not certain how FAA would tether to them, however if FAA does bind, it may be through a Grb adapter protein at the putative SH2/SH3 domains. Therefore, FAA may act as a scaffold protein in gonadotroph cells to regulate α GSU transcription.

Chapter 5:

General Discussion

L β T2 cells are an *in vivo* derived cell line that represent fully differentiated gonadotrophs (Turgeon, Kimura et al. 1996). Pulsatile administration of GnRH induces synthesis of LH β mRNA and secretion of LH (Turgeon, Kimura et al. 1996) and FSH β mRNA expression is only induced with activin and GnRH (Graham, Nusser et al. 1999; Pernasetti, Vasilyev et al. 2001). Therefore, these cells mimicked *in vivo* expression of gonadotrophins, in an *in vitro* model system and hence were chosen for this project.

Differential display RT-PCR was used successfully, to identify GnRH regulated mRNA transcripts in L β T2 cells. This PCR based technique was developed by Liang et al to identify differentially expressed mRNAs (Liang and Pardee 1992). Since it is a PCR based technique, it has the advantage of being highly sensitive, so that low copy number transcripts are also included in the analysis. In fact, most of the mRNA transcripts identified were low copy number transcripts, and could only be detected by ribonuclease protection assays (RPA). Although high frequencies of false positive results have previously been reported, none were identified during the course of this project. This may be due to the low complexity of the system used, since L β T2 cells are an *in vitro* model of gonadotrophs and therefore, this system is free of other contaminating mRNAs derived from other cell populations found in tissue samples. Additionally, the GnRH pulse experiments were carried out in duplicate so that only those transcripts that were differentially expressed in both would be isolated.

The initial pulse regime used was designed to mimic GnRH pulse frequencies during the luteal and follicular phases. Although this regime identified numerous changes in mRNA expression levels and identified that myosin light chain mRNA levels were up-regulated during the luteal phase, other differences were difficult to interpret, therefore a simpler regime was adopted. This GnRH pulse regime identified that mRNA expression levels changed rapidly, within one hour of GnRH stimulation, and usually returned to pre-GnRH levels after 4 hours. The rapidity of this response suggest that the GnRH response is transmitted through a mitogen activated protein kinase pathway since Levi et al identified that MAPK activity was up-regulated within 1 hour of GnRH stimulation (Levi, Hanoch et al. 1998). The MAPK cascade can also induce transcription in α T3-1 cells and GnRH pulses are required to maintain activation of MAPK (Roberson 1995; Haisenleder, Cox et al. 1998).

The GnRH regulated mRNA transcripts that were identified included two ESTs, one found in the mammary gland and the other in the testes, the identity and function of these transcripts are currently unknown. Initially, confirmation of their expression pattern is required by RPA analysis before further characterisation. These clones (R3 and R18), could then be used to screen a cDNA library or used as templates for rapid amplification of cDNA ends (RACE), to generate full length clones that can be used in structural and functional studies. From the cDNA sequences, protein sequences can be determined. These can be used to predict and identify protein motifs or homology to other known proteins that may offer some clue as to the function of these proteins. The full length cDNAs could also be used to screen yeast 2-hybrid libraries as bait proteins to identify interacting

partners, and transfected into L β T2 cells to identify a possible role for these proteins in regulating gonadotrophin synthesis.

The other GnRH regulated mRNA transcripts were myosin light chain (MLC), a putative tyrosine phosphatase and Fanconi's Anaemia complementation group A (FAA). MLC forms part of myosin-I, a myosin motor that is involved in intracellular vesicle transport (Cheney and Mooseker 1992; Mermall, Post et al. 1998). It is involved in regulating the activation of myosin-I by myosin light chain kinase (MLCK), whose activation is also regulated by GnRH (Rao, Paik et al. 1997). Western blot analysis of MLC could be carried out to show if the protein levels were different in the luteal and follicular phases, which would further validate the hypothesis that MLC is involved in luteal phase exocytosis of the LH granules. Confocal microscopy could be used to show if MLC co-localised with the LH secretory granules in L β T2 cells, in the presence or absence of GnRH, and under conditions that mimicked the luteal or the follicular phase.

Activation of the GnRH receptor by GnRH has been shown to up-regulate the activity of a tyrosine phosphatase in pancreatic tumour cells and suppress the tyrosine kinase response induced by epidermal growth factor (EGF) by dephosphorylating the GnRH receptor (Liebow, Lee et al. 1990; Liebow, Lee et al. 1991). Tyrosine kinases are involved in regulating growth and differentiation of cells however *in vivo*, gonadotrophs are fully differentiated and do not undergo further mitosis. Epidermal growth factor receptor, a tyrosine kinase, has been shown to up-regulate LH β mRNA expression levels in anterior pituitary cells, and recent evidence suggests that it may also up-regulate translation in α T3-1 cells (Armstrong and Childs 1997; Sosnowski, Mellon et al. 2000). Therefore tyrosine kinases may be involved

in regulating LH synthesis, and tyrosine phosphatases regulate tyrosine kinases in a mechanism similar to negative feedback regulation (Alberts, Bray et al. 1994). So, the tyrosine phosphatase identified may have an indirect role in regulating LH β synthesis. It may be that the tyrosine phosphatase is involved in controlling the GnRH response, down-regulating it until the next GnRH pulse. To test this, a tyrosine phosphatase inhibitor such as sodium orthovanadate could be added to L β T2 cells before stimulating the cells with GnRH. Then either a nuclear run on assay to measure LH β transcription rate or an RPA to measure LH β mRNA levels could be performed, and the results compared with L β T2 cells that had not been treated with the tyrosine phosphatase inhibitor. If the LH β mRNA levels, or the rate of transcription was increased in cells treated with sodium orthovanadate, this would provide further evidence for the role of tyrosine kinases and phosphatases in regulating LH β transcription. It would also provide evidence of another pathway involved in the differential regulation of gonadotrophin gene expression.

DD-RT-PCR has shown that FAA is present in gonadotrophs, and is regulated by GnRH. However, the ambiguity between the DD-RT-PCR mRNA expression profile and the RPA profile needs to be clarified. The identification of a penta zinc finger (zfp276) that is found at the 3' end of FAA in the opposite orientation (Wong, Alon et al. 2000), requires further analysis since it may be this transcript that is up-regulated in response to GnRH. To test this, zfp276 will need to be isolated, cloned and used in RPA analysis to identify if its mRNA expression profile matches the DD-RT-PCR expression profile. If zfp276 mRNA expression levels are up-regulated in response to GnRH, it would suggest that zfp276 may have an opposite effect to FAA and

up-regulate α GSU promoter activity. To test this, zfp276 could be co-transfected into L β T2 cells with LH β , FSH β and α GSU promoter constructs and into α T3-1 cells with the α GSU promoter constructs. This would identify if zfp276 can regulate promoter activity in a cell-line and promoter specific manner and would suggest that zfp276 and FAA lie on a common pathway. FAA down-regulates α GSU promoter activity and has no effect on LH β promoter activity. It is uncertain if FAA affects FSH β promoter activity, although it would seem unlikely based on the specific action of FAA, which has already been discussed. However, this must be addressed by co-transfection of FAA with the FSH β gene promoter. FAA appears to have two different effects on the α GSU promoter, a GnRH inhibitory effect on the longer promoter that is specific to L β T2 cells, and a basal stimulatory effect on the shorter promoter, which is common to L β T2 and α T3-1 cells. This suggests that a factor common to both L β T2 and α T3-1 cells is involved in basal regulation of α GSU transcription, and a factor that is specific to L β T2 cells is involved in GnRH regulated α GSU transcription. To identify these factors, the two regulatory regions on the α GSU promoter have to be further localised. So, a series of α GSU promoter resections could be made and co-transfected with FAA to isolate the inhibitory region on the -480bp α GSU promoter. Electrophoretic mobility shift assays (EMSAs) using this region could be performed to identify if a protein binds directly to this region in the presence or absence of GnRH. To identify if this protein is FAA a supershift assay, using a FAA antibody, could be performed. If however FAA does not bind this region, then a number of techniques could be used to identify the protein that does bind. The DNA with the protein binding

region could be covalently linked to biotinylated beads and mixed with crude cytosolic or nuclear extracts, to isolate and separate proteins that bind to this DNA fragment using a streptavidin coated matrix column. Alternatively, the DNA fragment containing the protein binding region could be used to screen a cDNA expression library. The isolated cDNA could then be *in vitro* transcribed and translated and the resulting protein could be analysed. Additionally, this protein could be used to identify if it interacts with FAA by yeast 2-hybrid analysis. A similar approach could be used to localise the region on the -120bp α GSU promoter that up-regulates the basal transcription rate of the promoter. Initially, the homeobox domain binding site on the -120bp α GSU promoter should be mutated to demonstrate if the FAA response is mediated through this site. If this was the case, promoter resections would be unnecessary and the homeobox domain binding site could be used in the experiments described above.

A western blot of untreated and GnRH treated L β T2 nuclear and cytosolic preparations could also be performed to identify if FAA shuttles between the nucleus and cytoplasm in response to GnRH. Additionally immunocytochemistry could be performed on L β T2 cells that were either left untreated or treated with GnRH. This would identify if FAA is translocated from the nucleus in response to GnRH stimulation. This would provide evidence that FAA could be acting as a shuttling protein, and strengthen the hypothesis that it may be acting as a scaffold protein.

It is not known if any of the other FA proteins are present in gonadotrophs. To determine if the other FA proteins are present, RT-PCR using primers designed to FAG and FAC could be performed. If the other FA proteins are present, it is most likely that FAA acts as part of a complex. If this is the case,

then FAC is the likely candidate for interactions with other proteins since there is evidence that FAC can interact with a number of different effectors (Hoatlin, Zhi et al. 1999; Cumming, Lightfoot et al. 2001; Pang, Keeble et al. 2001). Therefore FAC and FAG could be cloned from L β T2 cells and used in transfection assays to determine if they have any effect on the α GSU promoter either alone or together with FAA. If the other FA proteins are not present, then it suggests that FAA may be involved in a novel pathway that regulates the α GSU promoter. Screening a yeast 2-hybrid library may also identify other proteins that have not previously been described and it may also identify if FAA interacts with zfp276.

The FAA regulatory region has been provisionally mapped between 1aa and 322aa. To confirm this, a further transfection experiment should be carried out using the construct described in Fig. 4.2.5a that is currently under construction. This construct is missing the first 322aa of the N-terminus, which appears to be responsible for mediating repression of α GSU promoter activity, therefore this construct should be unable to repress the GnRH regulated activity of the α GSU promoter. This would identify if this region is necessary for repression. Lightfoot et al identified that the region between 36 and 250aa was crucial for nuclear localisation, this region has significant homology with Ste5 and it is hypothesised that this region may be responsible for mediating repression. Therefore, two further FAA deletion constructs could be created; one that contained the amino acid residues between 36aa and 322aa, and the other containing the first 36aa, and the NLS. Co-transfection of these constructs with the -480bp promoter would determine if the repressor function was mediated through the NLS and

therefore FAG/FAC interaction, or through this Ste5-like region via an as yet unknown protein interaction.

The aim of this thesis and this project was to identify GnRH regulated factors that may be involved in differentially regulating gonadotrophin biosynthesis and secretion, and add to the knowledge of the downstream effects of GnRH. This thesis has described a number of factors that are rapidly regulated by GnRH, which are involved in regulating synthesis and secretion of the gonadotrophins. Additionally, this work has shown that DD-RT-PCR is a reliable technique to use to identify changes in mRNA expression profiles in L β T2 cells, since the mRNA expression profiles of those transcripts tested have been confirmed. Therefore the mRNA expression profiles identified by DD-RT-PCR are true representations of the actual mRNA expression patterns in the cell, although further clarification of the mRNA expression profile of FAA needs to be carried out, since the expression profile on the DD-RT-PCR gel could be representative of zfp276. Since the GnRH regulated mRNA transcripts identified by DD-RT-PCR are involved in secretion, signalling and transcription of the gonadotrophins, then the L β T2 cell line has also proved to be a good *in vitro* model of gonadotrophs. Furthermore there is previous evidence showing that a few of these factors may be GnRH regulated, therefore all the machinery involved in the synthesis and secretion of LH and FSH is present in these cells and respond as expected to GnRH. The novel discovery that GnRH regulates FAA, which in turn regulates α GSU transcription provides evidence for a unique role for FAA in the gonadotrophs and may provide further insight into the role of this protein out-with the gonadotroph cell.

Appendix I

This appendix contains a list of the commercial suppliers used during the course of this thesis. It also contains the addresses of the academic sources used.

Academic Sources

P.Brown
MRC Human Reproductive Sciences Unit
Centre for Reproductive Biology
37 Chalmers Street
Edinburgh
EH3 9ET
UK
Tel: 0131 2292575
Fax: 0131 2285571
email: p.brown@hrsu.mrc.ac.uk

A.S.McNeilly
MRC Human Reproductive Sciences Unit
Centre for Reproductive Biology
37 Chalmers Street
Edinburgh
EH3 9ET
UK
Tel: 0131 2292575
Fax: 0131 2285571
email: a.mcneilly@hrsu.mrc.ac.uk

P.Mellon
The Salk Institute
PO Box 85800
San Diego
CA 92138-9216
USA

H.J. van de Vrugt
Department of Clinical Genetics and Human Genetics
Free University Medical Centre
Amsterdam
The Netherlands

Commercial Suppliers

AB Gene (UK) Ltd

Ambion (UK) Ltd
Amersham-Pharmacia (UK) Ltd
AMS Biotechnology (UK) Ltd
Anachem (UK) Ltd
Applied Biosystems (UK) Ltd
BDH (UK) Ltd
Becton Dickinson Laboratories (UK) Ltd
Beckman Coulter (UK) Ltd
Bioquote (UK) Ltd
Biorad Laboratories (UK) Ltd
Calbiochem (UK) Ltd
Cambio (UK) Ltd
Clontech (UK) Ltd
Flowgen (UK) Ltd
Gene Therapy Systems (UK) Ltd
Genetic Research Instrumentation Ltd
Gibco BRL, Life Technologies Ltd
Greiner Labortechnik Ltd
Hybaid (UK) Ltd
Invitrogen (UK) Ltd
Kendro (UK) Ltd
Life Technologies (UK) Ltd
Millipore (UK) Ltd
Nalgene (UK) Ltd
New England Biolabs (UK) Ltd
Novagen, CN Biosciences (UK) Ltd
Promega (UK) Ltd
Qiagen (UK) Ltd
Roche Diagnostics (UK) Ltd
Sigma-Aldrich Company (UK) Ltd
Sigma-Genosys (UK) Ltd
Stratagene (UK) Ltd

Appendix II

Anchored 3' primers

24 μ M T₁₂VA (containing 8 μ M each of the following):

T12AA	5'-TTTTTTTTTTTTTAA-3'
T12CA	5'-TTTTTTTTTTTTTCA-3'
T12GA	5'-TTTTTTTTTTTTTGA-3'

24 μ M T₁₂VC (containing 8 μ M each of the following):

T12AC	5'-TTTTTTTTTTTTTAC-3'
T12CC	5'-TTTTTTTTTTTTTCC-3'
T12GC	5'-TTTTTTTTTTTTTGC-3'

24 μ M T₁₂VG (containing 8 μ M each of the following):

T12AG	5'-TTTTTTTTTTTTTAG-3'
T12CG	5'-TTTTTTTTTTTTTCG-3'
T12GG	5'-TTTTTTTTTTTTTGG-3'

Random upstream primers

5 μ M for DD-RT-PCR, 20 μ M for re-amplification

R1	5'-GGA ACTCCGT-3'
R2	5'-GGCAAGTCAC-3'
R3	5'-CCTCCGTAAG-3'
R4	5'-AGGACCGCTA-3'
R5	5'-CGGACCCTGG-3'
R6	5'-TAACTAACTC-3'
R7	5'-TACAACGAGG-3'
R8	5'-TGGATTGGTC-3'
R9	5'-TGGTAAAGGG-3'
R21	5'-AGTCAGCCAC-3'
SX	5'-GCGACCCATG-3'
TK	5'-CTTGATTGCC-3'
A1	5'-ACAGAGCACA-3'
A2	5'-ACGTATCCAG-3'
MAX1	5'-GAGCATATCC-3'

MAX2
MAX3

5'-CACAGCTTGC-3'
5'-CCACAGAGTA-3'

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